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(54) Title: Vpr AND Vpx PROTEINS OF HIV

(57) Abstract

This invention relates to a biologically active peptide fragment of the Vpr protein of human immunodeficiency virus, to pharmaceutical compositions comprising these peptides or biologically active analogues thereof, to antagonists of the peptides, and to pharmaceutical compositions comprising these antagonists and to therapeutic and screening methods utilising compounds and compositions of the invention. In one preferred embodiment, the invention provides an antagonist of the Vpr protein of human immunodeficiency virus (HIV), or of a biologically active fragment or analogue thereof, comprising at least one amino acid sequence motif selected from HFRIG and HSRIG which has the ability to inhibit one or more activities mediated by Vpr, selected from the group consisting of growth arrest, cell replication arrest, cytotoxicity, cytoskeletal disruption, and effects on the endoplasmic reticulum. The invention also relates to use of Vpr protein, or a biologically active fragment or analogue thereof comprising the consensus sequence, in treatment of conditions mediated by cellular proliferation or caused by eukaryotic pathogens.

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Vpr and Vpx proteins of HIV

This invention relates to a biologically-active peptide fragment of the Vpr protein of human immunodeficiency virus, to pharmaceutical compositions comprising these peptides or biologically-active analogues thereof, to antagonists of the peptides, and to pharmaceutical compositions comprising these antagonists and to therapeutic and screening methods utilising compounds and compositions of the invention.

Background of the Invention

HIV-1, the causative agent of AIDS, is a complex retrovirus like other primate lentiviruses, having genes *tat*, *rev*, *vif*, *vpr*, *vpu*, and *nef* that are not found in simple retroviruses. While the functions of *tat* and *rev* are fairly well understood, the remainder, often referred to as auxiliary genes because they are not essential for *in vitro* infectivity of the virus, have poorly understood roles in pathogenesis.

HIV-1 viral protein R (Vpr) (Wong-Staal *et al*, 1987) is a virion-associated protein (Cohen *et al*, 1990a; Yuan *et al*, 1990). There have been reports that HIV-1 Vpr is a weak transcriptional activator (Ogawa *et al*, 1990; Cohen *et al*, 1990b) and that it binds to the HIV-1 Gag protein (Lu *et al*, 1993; Paxton *et al*, 1993; Lavallee *et al*, 1994). Although Vpr is not essential for virus replication in established cell lines (Dedera *et al*, 1989; Cohen *et al*, 1990b), there is evidence to suggest that it may have a critical function for viral replication in primary macrophages (Balotta *et al*, 1993; Matsuda *et al*, 1993). Because of its association with the virion, it has been suggested that Vpr has an early role in HIV-1 infection, possibly in penetration or uncoating of the virus (Cohen *et al*, 1990a; Yuan *et al*, 1990; Yu *et al*, 1990).

Vpr is one of the most highly conserved proteins of HIV-1, and exists as Vpr and/or Vpx in all primate lentiviruses (Tristem *et al*, 1990; see Fig. 4A). Vpx is similarly virion-associated (Cohen *et al*, 1990a; Yuan *et al*, 1990; Yu *et al*, 1988, 1990, 1993). HIV-2 Vpr is essential for productive infection of human macrophages (Hattori *et al*, 1990), but like HIV-1 Vpr it is dispensible for replication in established cell lines (Dedera *et al*, 1989). Similarly HIV-2 Vpx is dispensible in established cell lines (Yu *et al*, 1988; Guyader *et al*, 1989; Hu *et al*, 1989) but is required for infection in fresh macrophages (Guyader *et al*, 1989; Yu *et al*, 1991), and augments viral infectivity in peripheral blood lymphocytes (Kappes *et al*, 1991). Perhaps most convincing of all, it has been observed that there is a drive *in vivo* for retention of an intact *vpr* reading frame and that mutations in *vpr* lead to a low virus burden in Rhesus monkeys (Lang *et al*, 1993).

We have cloned the Vpr gene in yeast, and have compared the effect of Vpr protein on haploid yeast cells with the effects of the proteins Vif, Vpu and Nef. We have surprisingly found that the Vpr protein has profound effects on cell growth, while the other proteins tested have no effect. The Vpr protein causes growth arrest, and this appears to be mediated by effects on the cytoskeleton. We have identified the portion of the Vpr protein which is critical for this activity. This critical portion comprises a conserved amino acid sequence motif, H(S/F)RIG, and peptides comprising this portion are active when added extracellularly to mammalian or yeast cells. Since the Vpr protein appears to have an early and possibly critical role in HIV infection, it represents a useful therapeutic target.

We have also found that the Vpr protein, and particularly the C-terminal sequence thereof, has a general antiproliferative effect on eukaryotic cells, and therefore is useful in the treatment of conditions mediated by cell proliferation.

Summary of the Invention

According to one aspect, the invention provides a method of treatment of HIV infection, comprising the step of administering to a subject in need of such treatment an effective amount of an antagonist of Vpr protein, or of a biologically active fragment or analogue thereof comprising at least one motif selected from HFRIG and HSRIG, thereby to prevent HIV infection, to prevent progression of HIV infection to symptomatic AIDS, or to alleviate the symptoms of AIDS.

Preferably the Vpr protein comprises at least one sequence selected from the group consisting of HSRIG, HFRIG, HSRIS, HFRAG, HIRAG, HLRAG, RSRKG, RSRIS and RSRIG,

In a second aspect, the invention provides an antagonist of the Vpr protein, or of a biologically active fragment or analogue thereof, as defined above, which has the ability to inhibit one or more activities mediated by Vpr, selected from the group consisting of growth arrest, cell replication arrest, cytotoxicity, cytoskeletal disruption, and effects on the endoplasmic reticulum. It is considered that such antagonists will be useful as therapeutic agents for treatment of HIV infection.

The invention also encompasses a pharmaceutical composition comprising as active component an antagonist of Vpr as defined above, together with a pharmaceutically-acceptable carrier.

The person skilled in the art will recognise that specific antibody, preferably monoclonal antibody, directed against Vpr or a biologically active fragment or analogue thereof, and antisense RNA or triple-stranded DNA which prevents expression of Vpr or of said biologically-active fragment or analogue, provide methods of inhibition of the activity of Vpr, and consequently are within the scope of this invention. Methods for production of monoclonal antibodies against a given peptide sequence, and methods for inducing antisense RNA or triple-stranded DNA production in a target cell are well known in the art. For

example, a vpr gene in which the region encoding C-terminal portions of the Vpr protein has been replaced by an inhibitory antisense sequence or by a sequence which encodes an inhibitory peptide could be used for gene therapy of HIV infection or of AIDS.

In a third aspect, the invention provides a method of screening compounds suspected of being useful as antagonists of Vpr protein , or of a biologically active fragment or analogue thereof as defined above, comprising the step of measuring the effectiveness of a test compound in inhibiting the activity of Vpr in an assay of a biological activity selected from the group consisting of growth arrest, cell replication arrest, cytotoxicity, cytoskeletal disruption, and effects on the endoplasmic reticulum, as herein described.

Our results indicate that the Vpr protein or biologically active fragment or analogue thereof has activities which can be attacked at either the intracellular or extracellular level, and therefore both types of biological activity are within the scope of the invention.

According to a fourth aspect, the invention provides a vaccine for prevention of HIV infection or for alleviation of the effects of HIV infection, comprising human immunodeficiency virus-1 or human immunodeficiency virus-2 from which the portion of the HIV genome encoding at least the C-terminal 21 amino acids of the Vpr sequence has been deleted, together with a pharmaceutically-acceptable carrier. Preferably the portion of said genome encoding at least the C-terminal 33 amino acids of the Vpr sequence has been deleted. Even more preferably both a portion of the genome encoding the C-terminal region of the Vpr sequence and the portion of the HIV genome encoding the N-terminal of the Nef gene have been deleted. Relevant portions of the Nef gene are described in our co-pending Patent Application No. PCT/AU94/00254 (WO 94/26776), filed 18 May 1994.

According to a fifth aspect, the invention provides a method of treatment of a disease mediated by cell proliferation, comprising administration to a mammal in need of such treatment of an effective amount of Vpr protein, or of a biologically active fragment or analogue thereof comprising at least one sequence of the consensus sequence disclosed herein, thereby to inhibit proliferation of cells mediating the said disease.

Diseases mediated by cell proliferation include, but are not limited to, cancer, leukaemias and psoriasis.

Proliferating cells are highly calcium dependent. We have found that the anti-proliferative effect of Vpr peptides is blocked by an inhibitor of Ca^{2+}-channel transport. Therefore in this aspect of the invention, the Vpr protein or fragment or analogue thereof may optionally be used in conjunction with an enhancer of Ca^{2+}-channel transport.

According to a sixth aspect, the invention provides a method of treatment of a disease caused by a pathogen, comprising the step of administering to a mammal in need of such treatment of an effective amount of Vpr protein, or of a biologically-active fragment or analogue thereof comprising at least one sequence of the consensus sequence disclosed herein. Diseases caused by bacteria, parasites, yeasts, or fungi are all within the scope of this aspect of the invention.

According to a seventh aspect, the invention provides an agent for delivery of a pharmaceutically active substance to a cell membrane or to the interior of a cell, comprising a Vpr peptide or a biologically active fragment or analogue thereof comprising at least one amino acid sequence motif selected from HFRIG and HSRIG, said peptide, fragment or analogue being linked to said pharmaceutically active substance.

The invention also provides a method of delivery of a pharmaceutically active substance to a cell membrane or to the interior of a cell, comprising the step of

contacting said cell with an agent as defined above.

The active substance may be any chemical entity capable of being linked to a peptide, and in particular may be a peptide or a nucleotide. Coupling may be effected by any convenient means, for example chemical coupling using agents such as carbodiimide. Where the active substance is a peptide, the Vpr peptide and the pharmaceutically active peptide may be synthesised together by recombinant means as a fusion protein. The person skilled in the art will be aware of a variety of suitable pharmaceutically active agents which could be delivered in this way, and of methods whereby they may be linked to the Vpr protein. Such a person will also be aware of methods suitable for testing whether the linkage has been effective, and whether the agent retains the desired pharmaceutical activity.

In preferred embodiments of the invention, the Vpr protein is a fragment comprising at least the C-terminal 21 amino acids of the Vpr sequence, more preferably at least the C-terminal 33 amino acids of the Vpr sequence.

It will be clearly understood that Vpr protein or its biologically active fragments linked to a carrier or fusion protein, such as glutathione-S-transferase (GST), are within the scope of the invention. It will be further understood that recombinant, synthetic and naturally-derived Vpr protein and fragments and analogues thereof are within the scope of this invention.

While the description herein relates specifically to Vpr protein of HIV-1, as described above HIV-2 also possesses a Vpr protein, and it will be clearly understood that the invention is equally applicable to the Vpr protein and the equivalent of the H(S/F)RIG motif derived from HIV-2, and to the Vpx protein of other lentiviruses.

Throughout this specification, the single letter code of abbreviations for amino acids is used. S/F indicates that either S or F may be present.

Detailed Description of the Invention

The invention will now be described in detail by way of reference only to the following non-limiting examples, and to the drawings, in which:

Figure 1 illustrates the scheme employed for cloning of the *vpr* gene for expression in yeast;

Figure 2 shows the effect on growth of expression of HIV-1 auxiliary proteins in yeast;

Figure 3 shows the morphological changes in yeast cells expressing Vpr.

Copper-induced yeast cells were examined by light microscopy. A DY150 [pYEULCBX] control transformant is shown in the top panel (A) and a typical large DY150 [pYEULCBX.Vpr] transformant is shown on the bottom panel (B). The bar represents 10 μm.

Figure 4 shows the results of analysis of induced cells by flow cytometry;

Figure 5 shows the identification of the toxic region in Vpr;

A series of Vpr constructs is indicated. The region of Vpr produced by the construct is represented by pale blocks, while dark blocks represent GST; GST is not drawn to scale. The position of *Bam*HI (B), *Eco*RI (E) and *Sal*I (S) sites relative to the protein sequence is indicated. H(S/F)RIG, shown in bold, is encoded by sequences on either site of the *Sal*I site. Growth of the transformed yeast cells producing these proteins is recorded in the right column.

Figure 6 shows the relationship between HIV-1 Vpr and proteins from HIV-2, SIV and yeast.

A. Alignment of HIV-1 Vpr with Vpr relatives.

Vpr and Vpx proteins are aligned in their entirety. Sequences are derived from HIV-1 Vpr NL4-3 (Adachi *et al*, 1992), HIV-2ROD (Clavel *et al*, 1992) and SIVmac239 (Regier and Desrosiers, 1990). Regions with three or more identical amino acids are shaded.

B. Alignment of Sac1p and HIV-1 Vpr.

The entire amino acid sequence of Vpr is aligned with amino acids 77-176 of Sac1p (Cleves *et al*, 1990). Identical residues are shaded, and the residues in the H(S/F)RIG motif are underlined.

Figure 7 illustrates the osmosensitivity of yeast expressing Vpr.

Peptide 1 NH_2-VTRQRRARNGASRS-COOH

Peptide 2 NH_2-CRHSRIGVTRQRRARNGASRS-COOH

Peptide 3 NH_2-HFRIGCRHSRIGVTRQRRARNGASRS-COOH

Figure 8 illustrates the strategy used for the construction of mutant viruses.

Figure 9 shows the replication kinetics of mutant viruses in human PBMC, as measured by reverse transcriptase assay. A. Stimulated cells; B. Unstimulated cells

Figure 10 shows the effect of synthetic Vpr peptides on yeast colony formation. A, no peptide; B, Peptide 1; C, Peptide 2; D, Peptide 3.

Figure 11 summarises the results of the response of yeast cells to synthetic Vpr peptides. Treatment conditions are as described in Example 11.

Figure 12 shows the dose response relationship for Peptide 3. Peptide 3 was added to yeast cells over a concentration range and cells were assayed for colony formation.

Figure 13 shows the effect of yeast cell concentration on colony formation in the presence of a Vpr peptide. Various cell concentration were incubated in the presence of 5 μM Peptide 3 and cells were assayed for colony formation.

Figure 14 shows the results of flow cytometric analysis of propidium iodide (PI) uptake following incubation of yeast cells with synthetic Vpr peptides for 1 h. Samples are No peptide, Peptide 1, Peptide 2 and Peptide 3.

Figure 15 shows the results of flow cytometric analysis of propidium iodide uptake following incubation of yeast cells with Peptide 3 for various times.

Figure 16 shows the results of flow cytometric analysis of propidium iodide uptake in RC2a cells electroporated with synthetic Vpr peptides.

Figure 17 shows results obtained when mammalian cells were electroporated and analysed by flow cytometry for changes in cell structure as measured by forward and side scatters.

Figure 18 shows protection of yeast cells by TMB-8.

Figure 19 shows the association of FITC (fluorescein isothiocyanate)-labelled peptides with $CD4^+$ human cells measured by flow cytometry following (A) electroporation, and (B) extracellular addition without electroporation.

Figure 20 shows the association of FITC-labelled peptides without electroporation in *S. cerevisiae* yeast cells measured by flow cytometry.

Figure 21 shows the internalised FITC-labelled peptide 3 in human $CD4^+$ cells (A) and in yeast cells (B).

Figure 22 shows the genetic interaction between Vpr and Sac1p and actin.

Yeasts and bacteria

Yeast strains employed in this study were *Saccharomyces cerevisiae* strain DY150 (*MATa ura3-52 leu2-3, 112 trp1-1 ade2-1 his3-11 can1-100*), *Candida albicans* clinical isolate JRW5, *Candida glabrata* strain L5 (*leu*), *Kluyveromyces lactis* strain MW-98-8c (*α uraA arg lys*) and *Schizosaccharomyces pombe* strain SpULA (*ade6-704 ura4-D18 leu1-32*)h^-. Strains were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). An *Escherichia coli* strain TG1 Δ(*lac-proAB*) *supE thi hsdΔ5* F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*] was also employed for toxicity studies and plated onto 2xYT medium (1.6% tryptone, 1% yeast extract, 0.5%

NaCl).

Mammalian cells

Two CD4+ cell lines, RC2a and Jurkat, were maintained in RPMI-1640, containing 10% heat inactivated foetal calf serum (HIFCS). Mononuclear cells were isolated from HIV-1 seronegative blood obtained from blood bank volunteers by a standard Ficoll/Hypaque density gradient method. The peripheral mononuclear leukocyte cells (PBMC) were stimulated with phytohaemagglutinin (PHA : 10 $\mu g/10^6$ cells) for 48 h at 37°C, and were washed and resuspended in IL-2 medium, containing RPMI-1640 medium with 10% HIFCS, 10% recombinant human Interleukin-2 (Boehringer Mannheim), 5 mM Hepes, 0.1% sodium bicarbonate, 25 μg/ml glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml polybrene (Sigma) and 1:1000 anti-interferon (Miles).

Example 1 Molecular Cloning of HIV Genes

The HIV-1 genomic clone pNL4-3 was used as the source of HIV-1 genes for amplification by polymerase chain reaction (PCR). pNL4-3 (Adachi *et al*, 1986) was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, National Institutes of Allergy and Infectious Diseases, NIH (Adachi *et al*, 1986). The cloning of the *vpu* and *nef* genes has been described previously (Macreadie *et al*, 1992, 1993). The scheme used for cloning of *vpr* is described in Figure 1, while the cloning of *vif* employed PCR and similar known procedures. *vpr* was amplified from the HIV-1 genomic clone pNL4-3 (Adachi *et al*, 1986) using PCR and the primers shown. The PCR product was cleaved with *Bam*HI + *Sma*I and cloned into the yeast - *E. coli* shuttle vector pYEULCBX (Macreadie *et al*, 1992), and digested with *Bam*HI + *Eco*RI (T4 polished) to produce pYEULCBX.Vpr. In like fashion the other HIV-1 genes, *nef*, *vpu* and *vif* were also cloned into pYEULCBX to produce pYEULCBX.Nef27 (Macreadie *et al*, 1993), pYEULCBX.Vpu (Macreadie *et al*, 1992) and pYEULCBX.Vif.

These plasmids were designed to direct the copper-inducible production of Nef, Vpu and Vif, respectively. *vif* primers were 5' GCTCCGGATCCATGGAAAACAGATGGCAGG and 5' CGCCCGGGAGCTCTAAAAGCTCTAGTGTCC. The *vif* PCR product was cloned as a *Bam*HI-*Sma*I fragment. *Bam*HI and *Sma*I cloning sites in the primers (above) are underlined, while sequences in italics represent the *vif* start and stop codons. All amplified DNA was sequenced to verify the absence of errors. Cloning of *nef*, *vpr*, *vpu* and *vif* genes into the yeast expression vector pYEULCBX was designed to direct the copper-inducible production of Nef, Vpu and Vif, respectively.

Example 2 Endogenously-Expressed *vpr* Protein Causes Growth Arrest in Yeast

In this study we expressed *vpr* in yeast in order to discern its functions. At the same time, as part of a general examination of the effects of the HIV-1 regulatory proteins on simple cellular functions, we also produced Vif, Vpu and Nef in haploid yeast and looked for their effects on cell growth. This was achieved by cloning *vpr* and the genes of other HIV-1 auxiliary proteins into the expression vector pYEULCBX to produce pYEULCBX.Vpr (see Fig. 1), pYEULCBX.Nef27 (Macreadie *et al*, 1993), pYEULCBX.Vpu (Macreadie *et al*, 1992), and pYEULCBX.Vif (this study).

Strain DY150 (*MAT*a *ura3*-52 *leu2*-3,112 *trp1*-1 *ade2*-1 *his3*-11 *can1*-100; Macreadie *et al*, 1993), obtained from Dr David Stillman at the University of Utah Medical Center, was transformed with the above yeast vectors plus vectors for the copper-inducible production of glutathione S-transferase (GST) and GST fused to Vpr.DY150 was grown on YEPD medium (1% yeast extract, 2% peptone, 2% glucose). Yeast cells were transformed by the electroporation procedure of Becker and Guarente (1991) and transformants were grown on minimal selective medium containing 20 μg/ml histidine, adenine and tryptophan and solidified, when

required, with 3% Phytagar™ (Gibco). Expression was induced by the addition of $CuSO_4$ to the amounts indicated, and growth was assayed. Transformants were suspended in sterile water and dropped out on to plates for growth at 28°C. The results are illustrated in Figure 2, which shows SD plates (0.67% yeast nitrogen base (Difco), 2% glucose) containing 0.25 mM $CuSO_4$, 20 μg/ml histidine, adenine and tryptophan and solidified with 3% Phytagar™ (Gibco). The proteins produced by the transformants are indicated.

As shown in Figure 2, profound effects on cell growth were caused by the Vpr protein, while the other HIV-1 proteins tested had no effect on vegetative cell growth. Low levels (0.25 mM) of $CuSO_4$ caused total growth arrest in cells expressing Vpr (Fig. 2), while no adverse effects were caused by the other proteins even with induction levels as high as 1 mM $CuSO_4$. The effect of Vpr was unrelated to copper toxicity, since with no added $CuSO_4$, where basal expression from the *CUP1* promoter is 5% of the induced level (reviewed in Macreadie *et al*, 1994), Vpr transformants grew at a slower growth rate than control transformants.

The Vpr toxicity was found to be due to growth arrest, not killing, since induced cells, even after 24 hours in the presence of the inducer, formed colonies when plated on media with no added copper. The DY150 [pYEULCBX.Vpr] transformant colonies grown up from the assay were considerably smaller than DY150 [pYEULCBX] transformant colonies. These "small" colonies grew like the parental DY150 [pYEULCBX.Vpr] transformant upon subsequent culture without added copper (data not shown), indicative of a cell cycle arrest after induction of Vpr synthesis followed by eventual recovery and return to the normal cell cycle.

Example 3 Arrested Cells are Greatly Enlarged

Examination of cells by light microscopy indicated that induced cells producing Vpr had a grossly

altered morphology. As shown in Figure 3, Vpr-producing cells had a diameter of 16 μm, more than twice the diameter of the control DY150 [pYEULCBX] transformants grown under the same conditions. It appeared that most of the intracellular space in the large cells was devoid of structure and occupied by a single large organelle, possibly a vacuole. This suggests that the DY150[pYEULCBX.Vpr] transformants were arrested in growth before cell division.

Example 4 Flow Cytometry Analysis

Cells were analysed and sorted using a Coulter Epics® Elite flow cytometry. Illumination was with a 488 nm Argon ion laser, and forward angle light scatter (related to cell size) and side scatter were recorded. Cells were sorted on the basis of forward angle light scatter. Live cells were gated by propidium iodide exclusion, indicated by absence of fluorescence emission at greater than 600 nm following staining with 2 μg/ml propidium iodide.

Induced yeast cells were analysed by flow cytometry forward angle light scatter (proportional to cell size) in order to assess the proportion of altered cells. The results are shown in Figure 4, and confirmed that in the cell population Vpr transformants exhibited a greater degree of forward light scattering, indicative of their larger size. Populations, containing over 50,000 cells, are for DY150 [pYEULCBX] and DY150 [pYEULCBX.Vpr] as indicated.

Example 5 Location of Sequences Causing Growth Arrest Includes H(S/F)RIG Repeated Motifs

The sequences responsible for causing the growth arrest were identified by testing various portions of the Vpr protein for effects on cell growth. Since Vpr fused to glutathione S-transferase (GST) also caused a growth arrest (Fig. 2; construct GST-Vpr, Fig. 5), we also produced a

series of GST fusion proteins in the yeast GST-fusion vector, pYEULCGT (Ward *et al*, 1994).

Deletion of the last 33 amino acids of Vpr, encoded by an *Eco*RI fragment (constructs VprBE and GST-VprBE, Fig. 5), relieved the growth arrest, while the addition of this portion of Vpr to GST (construct GST-VprEE, Fig. 5) caused a growth arrest, indicating that this domain was responsible for the growth arrest. A partial growth arrest was also seen with the addition of just the last 21 amino acids of Vpr to GST (construct GST-VprSE, Fig. 5).

In each case the growth arrest correlated with cell enlargement, as judged by flow cytometry analysis and light microscopy. Significantly, this C-terminal sequence is the region lacking in many laboratory HIV-1 isolates that encode a truncated *vpr* gene product of 73 amino acids due to a T insertion (Yuan *et al*, 1990; Ogawa *et al*, 1990; Lavallee *et al*, 1990). The Vpr in these isolates does not associate with virions (Ogawa *et al*, 1990), presumably because of the truncation. Our findings confirm the importance of the same C-terminal region, but for another reason. This growth arrest in yeast may be linked to AIDS pathogenesis.

The region of HIV-1 Vpr that causes cell growth arrest has been compared with known Vpr relatives, the closest relative being the SIV Vpr followed by HIV-2 Vpr, and then Vpx proteins (Fig. 6A). The sequence comprises 33% arginine, a much higher arginine content than that found in comparable portions of Vpx proteins. It is notable that there is conservation of a repeated motif, H(S/F)RIG, in Vpr species. The motif is present at amino acids 72-75 (encoded in the *Eco*RI-*Sal*I fragment), and at amino acids 78-82 (encoded in the *Sal*I-*Eco*RI fragment). The greater toxicity caused by the fragment encoding two copies may indicate a copy number effect or possibly a conformational effect.

In a search for a cellular relative to Vpr using the program ALIGN, we found that a yeast protein, Saclp (Cleves *et al*, 1989), has the most significant sequence similarity of cellular proteins listed in the Genbank database (release 82.0). In the alignment of Saclp and Vpr (Figure 6B) it can be seen that Saclp has 60% identity in the H(S/F)RIG motifs including the terminal Gs, the part of the motif that is totally conserved in Vpx as well. Over the entire alignment there are 32% identical and 45% similar amino acids.

Example 6 Peptide Synthesis

The peptides produced were as follows:

Peptide 1	NH_2-VTRQRRARNGASRS-COOH
Peptide 2	NH_2-CRHSRIGVTRQRRARNGASRS-COOH
Peptide 3	NH_2-HFRIGCRHSRIGVTRQRRARNGASRS-COOH
Peptide 4	NH_2-HFRIGCRHSRIG-COOH
Peptide 5	NH_2-RHSRIGVTRQRRARNGASRS-COOH
Peptide 6	NH_2-IFRAGTRYFRRG-COOH

Peptides were synthesised on an Applied Biosystems 430A Peptide Synthesizer, using the FastMoc solid-phase technique in which α-amino groups were protected by base-labile Fmoc (9-fluorenylmethyloxy-carbonyl) groups. The shortest sequence was synthesised on to the resin, then approximately one third of the peptide/resin was removed from the reaction vessel. Synthesis was then continued on the remainder until the second peptide was assembled, at which stage half of the peptide/resin was removed from the reaction vessel. The third peptide was then assembled on to the remaining peptide/resin.

The arginine side chains were protected by *tert*-butyl groups and glutamic acid by the O-*tert*-butyl group. Couplings were achieved by using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium

hexafluorophosphate activation of amino acids and N-methylpyrrolidone as solvent. The peptides were cleaved from the resin with trifluoroacetic acid (plus phenol, ethanedithiol, thioanisole and water as scavengers). The peptides were dialysed against electroporation buffer (0.213 g/l Na_2HPO_4, 0.068g/l KH_2PO_4, 93.1 g/l sucrose) (Wojchowski and Sytkowski, 1986) before electroporation.

For Peptides 4 to 6, protection was as follows: α-amino groups by base-labile 9-fluorenylmethloxycarbonyl (Fmoc) groups; arginine side chains by 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc); serine and threonine by *tert*-butyl groups; asparagine, glutamine, histidine and cysteine by trityl; and glutamic acid by the O-*tert*-butyl group. Couplings were achieved by using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluonium hexafluorophosphate (HBTU) activation of amino acids and N-methylpyrrolidone (NMP) as solvent. The peptides were cleaved from the resin with TFA, plus phenol, ethanedithiol, thioanisole and water as scavengers.

Example 7 H(S/F)RIG Motifs in Synthetic Peptides Cause Osmosensitivity

We further investigated the function of the H(S/F)RIG motifs using the synthetic peptides:

NH_2-VTRQRRARNGASRS-COOH

NH_2-CRHSRIGVTRQRRARNGASRS-COOH

NH_2-HFRIGCRHSRIGVTRQRRARNGASRS-COOH

produced in Example 6, that contain the penultimate 14, 21 and 26 amino acids, respectively, of Vpr. The H(S/F)RIG motif (underlined) is present at zero, one and two copies, respectively, within these peptides. These peptides were electroporated into yeast cells which were then analysed for osmosensitivity. Peptides, dissolved in electroporation buffer at 2 mg/ml, were electroporated into yeast cells using a Baekon 2000 (Saratoga, CA). Conditions

for the treatment in the Baekon 2000 were: 2^{11} pulses, 8 kV, 0.8 sec burst time, 100 μsec pulse time, 10 cycles, 1 mm gap between solution and upper electrode. The cuvettes contained 30 μl of yeast suspension in fresh YEPD growth medium plus 5 μl of Dulbecco's Phosphate-Buffered Saline and 5 μl of peptide solution. It was found necessary to achieve a kill of 60-80% in order to achieve uniform penetration of the surviving cells.

Cells were examined for osmosensitivity by plating onto YEPD medium and YEPD medium containing 1.2 M KCl, 1.8 M sorbitol or 0.9 M NaCl, and counting the numbers of colony-forming units, as described by Chowdhury *et al* (1992). Osmosensitivity was calculated by comparing the relative numbers of colony-forming units on the two media. All viable cells, including osmosensitive cells, grew on YEPD, but those that were osmosensitive did not grow on high osmotic strength media. The results, presented in Figure 7, show that cells treated with the peptide lacking an H(S/F)RIG motif were essentially unperturbed. However, the peptides containing H(S/F)RIG motifs caused osmotic sensitivity such that up to 50% of the cells were killed on high osmotic strength media. The effects were commensurate with the number of copies of H(S/F)RIG motif present, indicating a direct role for this sequence.

Example 8 Pathogenicity is Associated With the Sequence Containing H(S/F)RIG Motifs

The region of the Vpr protein containing H(S/F)RIG motifs may be correlated to the pathogenicity of human and simian immunodeficiency viruses. A brief compilation of sequences of Vpr and Vpx from human and simian immunodeficiency viruses is shown in Table 1. There is almost total conservation of the 12 amino acids containing two repeated H(S/F)RIG motifs in HIV-1, a highly pathogenic virus.

Seven simian immunodeficiency virus Vpr sequences show high conservation (two changes) of the sequence

containing the H(S/F)RIG motifs. However, the two sequences shown by the asterisk have poor conservation of the sequence (8 or 9 changes). Both the mandrill virus and the Sykes' monkey virus show poor sequence conservation, and are reported to cause asymptomatic infection (Hirsch *et al*, 1993; Tsujimoto *et al*, 1989).

In HIV-2 isolates there are between two and five changes from the reference sequence. HIV-2 is less pathogenic than HIV-1, and we believe that these changes may be a reason for the reduction in pathogenicity. Additionally the presence of Vpx may reduce pathogenicity. Matsuda *et al* (1993) showed that when Vpx replaced Vpr in HIV-1, the virus lost its infectivity. Thus we predict that any virus that produces Vpx may be expected to be less pathogenic than one which produces Vpr alone.

Table 1

H(S/F)RIG Motifs in Vpr Relatives

HIV-1	
NL43	HFRIGCRHSRIG
HAN	HFRIGCRHSRIG
MN	HFRIGCRHSRIG
ELI	HFRIGCQHSRIG
SC	HFRIGCRHSRIG
LAI	HFRIGCRHSRIG
SF2	HFRIGCQHSRIG
MAL	HFRIGCQHSRIG
OY1	HFRIGCQHSRIG
NDK	HFRIGCQHSRIS
NH52	HFRIGCQHSRMG
consensus	HFRIGCRHSRIG L Q MS
SIV	
SIVmac239	HFRGGCIHSRIG
SIVmac142	HFRSGCSHSRIG
SIVmac251	HFRGGCNHSRIG
SIVmacMNE	HFRGGCTHSRIG
SIVmmm H4	HFRSGCAHSRIG
SIVmmmPBJ	HFRGGCRHSRIG
SIV cpz	HFRLGCQHSRIG

Table 1 (cont)

	consensus	HFRGGCRHSRIG
		S I
		L S
5		T
		A
		Q
		N
	SIVmndGB1	HLAQGCDGTFRE *
10	SIVsykes	HFAAGCPQRTRY *
	HIV-2	
	ROD	HFRAGCGHSRIG
	D205	HFRAGCGHSRIG
	ISY	HFRAGCGHSRIG
15	NIHZ	HFRAGCGHSRIG
	CAM2	HFRAGCNHSRIG
	D194	HIRAGCDRSRKG
	GH1	HLRAGCNRSRIS
	ST	HFRAGCGRSRIG
20	BEN	HFRAGCNRSRIG
	consensus	HFRAGCGHSRIG
		I DR KS
		L N
	Vpx	
25	SIVmac239	HCKKGCRCLGEG
	SIVmac142	HCKKGCRCLGEG
	SIVmac251	HCKKGCRCLGEG
	SIVmacMNE	HCKKGCRCLGEG
	SIVmmm H4	HCKKGCRCLGEE
30	SIVmmmPBJ	HCKKGCRCLGGE
	consensus	HCKKGCRCLGEG
		GE
	HIV2 ROD	HVRKGCTCLGRG
	HIV2 D205	HYTKGCRCLQEG
35	HIV2 CAM2	HFKRGCTCLGGG
	HIV2 ISY	HFKKGCTCRGEG
	HIV2 NIHZ	HAKRDGTCLGGG
	HIV2 D194	HFKKGCTCLGRG
	HIV2 GH1	HFKRGCTCLGGG
40	HIV2 ST	HFKRGCTCLGGG
	HIV2 BEN	HFKRGCTCWGED

Table 1 (cont.)

	consensus	HFKKGCTCLGGG YRRD R WQRD VT R E A S
	SIVagm155	HFRCGCRRRQPF
	SIVagm 3	HFRCGCRRRQPF
	SIVagmTYO	HFRCGCRRRQPF
	consensus	HFRCGCRRRQPF

The overall consensus for the Vpr sequence, excluding those represented by the asterisk, is:

HFRIGCRHSRIG
I L QR MS
L G N
S I
S
T
A
Q
G
D

In summary, it appears that in the sequence HFRIGCRHSRIG, the residues underlined are invariant in Vpr. F can be I or L; the I can be L, G, S or M; the last G can be S. It should also be noted that the C between motifs is invariant.

Example 9 Replication Kinetics of Mutant Proviruses

Production and titration of virus culture

Half clones of the mutant and wild-type HIV DNA were co-transfected to HeLa cells (5 x 10^6 cells) in T25 flasks, by the Lipofectamine (GIBCO-BRL) method. PBMC (20 x 10^6 cells) were added 12 h after transfection and the cell free virus production was measured at regular intervals. The supernatant was harvested at maximum production of cell free virus and used as stock virus.

Titrations of virus stocks were done in 24 well Linbro plates, and the end point dilution was scored by both Reverse Transcriptase (RT) activity and visible cytopathic effect. RT assay in microtitre plates were performed according to standard methods.

Construction of mutant provirus

HIVNL 4.3 molecular clone (Adachi *et al*, 1986) was re-cloned as two half fragments into the pKP59 vector for the point mutation of the initiation codons of the *nef* and *vpr* genes. Mutant proviruses were constructed according to the procedures described in Figure 8, using the mutagenesis scheme summarized in Table 2.

Table 2

Mutagenesis Scheme

Gene	Oligo	Mutation position (nt)	Nucleotide changes	Final effect	Clone Into
Nef	N7 N8	8788 8829	ATG to AAG ATG to ATC	no nef expressed	pKP3EA
Vpr	V2	5559 5565	ATG to GTG CAA to TAA	no vpr expressed	pKP5SE
Vpr	VMM	5770 to 5804	deletion of 36 nucleotides	deletion of H(S/F)RIG motifs	pKP3EA

The HIV-1 molecular clone employed was pNL4-3. Because of instability of the full length clone in *E. coli*, half-clones were constructed in the low copy vector, pKP59, and stably maintained in *E. coli*. The 5' sequences were introduced as a *Stu*I-*Eco*RI fragment while 3' sequences were introduced as an *Eco*RI-*Avr*II fragment. These half-clones could be appropriately digested (*Xba*I+*Eco*RI fragment for the 5' clone and *Eco*RI+*Hae*II for the 3' clone) and the cut DNA introduced into mammalian cells where *in vivo* recombination restored the wild-type virus. To obtain

mutant virus appropriate segments of pNL4-3 were cloned into a phagemid, single-stranded DNA was produced, and second strand was synthesised in the presence of oligonucleotides, shown in Table 3, which were designed to introduce specific *vpr* and *nef* mutations.

Table 3

Mutagenic Oligonucleotides

Oligonucleotide	Code
5'-GGA TTT TGC TAT AAG AAG GGT GGC AAG-3'	N7
5'-GTA AGG GAA AGA ATC AGA CGA GCT G-3'	N8
5'-CAG AGG ACA GGT GGA ATA AGC CCC CAG AAG-3'	V2
5'-CTG CAA CAA CTG CTG TTT ATC *GTT ACT CGACAG AGG-3'	VMM

* indicates the site targetted for deletion in *vpr*

Following purification and verification of sequence changes the DNA was sub-cloned into the pKP59-half-clone, replacing the wild-type sequence with a mutant sequence. The mutant clones do not express Nef, do not express Vpr, or do not express either protein.

Infection of PBMC

Peripheral blood mononuclear cells were infected at a 0.01 multiplicity of infection (MOI), and the cell free supernatants were assayed daily for reverse transcriptase (RT) production by standard techniques.

In stimulated PBMC, mutant proviruses defective for the production of Nef or Vpr produced similar amounts of cell-free virus particles, which were in both cases considerably less than in the parent virus strain. The effect of Vpr on virus replication appears to be mediated by the H(S/F)RIG motifs, as shown in Table 4.

Table 4

Effect of Deletion of the H(S/F)RIG Motif on the Replication of Virus PBMC, as Measured by Cell-Free RT Activity

DAY (P.I)	HIVNL 4.3	VPR MOTIF (-)
3	7440	3878
7	269002	174735
10	219986	165945
15	109533	57493
17	93679	42692

A mutant provirus that was defective in the production of both Nef and Vpr was severely repressed in virus production, and showed delayed replication kinetics (Figure 9a). In unstimulated human primary cells, which closely resemble the *in vivo* cell population, both Nef and Vpr are indispensable for cell-free virus production (Figure 9b). The Vpr^- mutant produces smaller amounts of virus, the Nef^- mutant exhibits delayed replication kinetics, while the Nef^-Vpr^- double mutant shows no virus production. Therefore Nef and Vpr appear to act synergistically.

Example 10 H(S/F)RIG Motifs in Synthetic Peptides Cause Growth Arrest in Yeast

Peptides were dialysed against PBS and added at a final concentration of 2 μM to yeast cells suspended to a density of 10^6 cells/ml in a final volume of 200 μl water. After incubation for 1 h, 5 x 10^4 cells were spread on to solidified YEPD, and the plates were examined for colony growth after 40 hours incubation at 28°C. Peptide concentrations were determined by quantitative amino acid analysis of peptide solutions.

The addition of Peptide 2 or Peptide 3 caused the cells to completely lose colony forming ability, while the

addition of Peptide 1 had no effect (Figure 10). This compares with Example 5, in which we found a correlation of bioactivity, as assessed by osmosensitivity, with the intracellular presence of H(S/F)RIG motifs.

Therefore we further investigated peptides, such as Peptide 4, which contained only the H(S/F)RIG motifs, and which also caused some osmosensitivity, as shown in Figure 11. Peptide 4 also caused complete loss of colony forming ability. Peptide 5, which is like Peptide 2 but lacks the cysteine, also caused a considerable effect, suggesting that the cysteine was not essential for the activity, but that it did increase the activity, possibly due to a conformational effect.

We used Peptide 3 to establish a dose response relationship. Treatment of cells with a range of concentrations of Peptide 3 indicated that the lowest peptide concentration which induced complete growth arrest was about 1 μM, as shown in Figure 12. Concentrations down to 0.05 μM were partially effective, but below this concentration there was no effect.

Example 11 Blockage of the Growth Arrest Effect by Cell Mass

There was also an effect caused by cell concentration. At high cell concentrations the effectiveness of the peptides was limited. Figure 13 shows colony formation after treatment of yeast at a range of cell densities with 5 μM of Peptide 3. At cell densities up to 10^5 cells/ml a complete effect can be seen; however, at concentrations above 10^6 cells/ml no effect was observed. This suggests that about 10^6 molecules of peptide per cell are required for inhibition of colony formation. We have also observed that the effect may be abrogated by the presence of medium; for example the effect is greatly reduced in the presence of YEPD even at concentrations as low as 1/10 of normal strength.

Example 12 Effect of Synthetic Peptides on Other Microorganisms

We have investigated the effects of these peptides on the growth of several additional microorganisms, and the results are shown in Table 5.

Table 5

Effect of Peptide on Colony Formation of Bacteria, Budding Yeasts and Fission Yeast

	% "kill" after peptide treatment					
Peptide	*S. cerevisiae*	*C. albicans*	*C. glabrata*	*K. lactis*	*Sz. pombe*	*E. coli*
None	0	0	0	0	0	0
1	0	0	0	0	0	0
2	100	100	100	100	100	100
3	100	100	100	100	100	100
4	100	90	99	100	98	100
5	93	90	99	100	78	61

In *E. coli* and three budding yeasts, *Candida albicans*, *Candida glabrata* and *Kluyveromyces lactis*, and in the fission yeast *Schizosaccharomyces pombe*, the results were similar to those seen with *S. cerevisiae*. An effect was also seen on mammalian cells, in which the peptides inhibit the formation by RC2a cells of a lawn on a culture plate surface.

We treated *E. coli* with Peptides 1, 2 and 3 in the same way as yeast, except that the treated cells were suspended in 2% glucose/50 mN HEPS. The data in Table 5 show that peptides containing the H(S/F)RIG motif affect the viability of *E. coli*. However, we found that when *E. coli* was suspended in PBS and treated with these peptides, no loss of colony-forming ability was observed. It therefore appears that the activity is dependent on the medium used.

Example 13 Effect of Peptides on Yeast Cell Permeability

We examined cells with Fungolight Live/Dead Stain (Molecular Probes), and found that cells remained alive, ie. metabolically active, for several hours after the peptide treatments; however, they had lost colony forming ability. Thus the effect of the peptides seems to be to produce an irreversible growth arrest.

We further examined the peptide-treated cells by staining with propidium iodide, a vital stain, followed by flow cytometry. The results, shown in Figure 14, confirm that after 1 h there is a marked effect caused by Peptides 2 and 3. With Peptide 1 or no treatment, 95% of the cells take up little or no propidium iodide. With Peptides 2 and 3, fewer than half of the cells take up propidium iodide. Propidium iodide uptake is usually indicative of cell death or of membrane damage to the yeast. The analysis also shows that there is no significant cell lysis at this time.

Kinetic analysis of the effect of Peptide 3, shown in Figure 15, indicates that this effect is

immediate, with cells taking up propidium iodide within minutes of the peptide being added.

Example 14 VPR Peptides Kill $CD4^+$ Cells

We have used two $CD4^+$ cell lines, RC2a and Jurkat, to represent promonocytic and T lymphocytic cell lines respectively.

Electroporation of peptides

10 μg of peptide was added to 1 million $CD4^+$ cells which were suspended in 55 μl Baekon buffer (1.5 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 0.27 M Sucrose pH 7.0) and 10 μl of PBS (phosphate-buffered saline). Electroporation conditions in the Baekon 2000 Advanced Macromolecule Transfer System (San Francisco, CA) were 2^{11} pulses, 8 kV, 0.8 sec burst time, 62.5 μsec pulse time, 3 cycles, 85 mm gap between solution and upper electrode. Peptide-electroporated cells were resuspended in 1 ml RPMI-1640 10% HIFCS and incubated in a humidified 5% CO_2 incubator at 37°C.

Re-electroporation and pre-treatment of cells

Peptide-electroporated cells were harvested at 24 h for re-electroporation of the respective cells with peptides or without peptide, and the cells were analysed by flow cytometry after 24 h. One million cells were pretreated either with 0.5 nM TMB-8 hydrochloride ([8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate], HCl) or 0.5 μM prostaglandin E2 for 30 min before electroporation. Cells were maintained in the same concentration of the respective reagents after electroporation.

Preparation of cells for flow cytometry

One million cells were harvested and washed once in PBS at 1600 rpm for 5 min, and the pellet was resuspended in 200 μl PBS containing 2 μg propidium iodide in preparation for flow cytometry analysis. Cells were

analysed for a number of parameters 24 and 48 h after electroporation, using a Coulter Epics Elite Flow Cytometer. Forward and side scatters of a 488 nm argon ion laser were measured. Propidium iodide exclusion was measured by the absence of fluorescence emission at greater than 600 nm.

Figure 16 shows that Peptide 3 kills RC2a cells to a significant extent, compared to the mock electroporated cells. Peptide 2 killed a lower number of cells than did Peptide 3, and the results were comparable in both cell lines. However, Peptide 1, which lacks the H(S/F)RIG motif, does not affect these cell lines. The effect of Peptides 2 and 3 was enhanced by pretreatment with prostaglandin E2, and hence obviates the need for double electroporation. The effect of the peptide is modified by pre-treatment with the Ca^{2+}-channel blocker TMB-8 HCl. The H(S/F)RIG motif also influences the cell structure as measured by forward and side scatters, as shown in Figure 17. Peptide 3 has produced a right shift of both side and forward scatters compared to the mock electroporated and other peptides electroporated cells. This shows that Peptide 3 induces an increase in both cell size and cellular granularity.

Example 15 Blockage of Yeast Cell Growth Arrest by TMB-8 and High Ionic Strength

The addition of TMB-8 for 30 min prior to the addition of the peptides abrogates the effect of Peptide 3, as shown in Figure 18.

Various concentration of TMB-8 were preincubated with yeast cells for 30 min and then the cells were incubated in the presence or absence of 5 μM Peptide 3. Cells were then assayed for colony formation. With no TMB-8 there were no colonies formed. Low concentrations of TMB-8 gave protection to about 13% of cells.

TMB-8 caused some toxicity at high concentration, but at lower concentrations no toxicity was observed. At

these lower levels of TMB-8 Vpr peptides were not totally effective in inhibiting the colony forming ability of yeast cells.

Sodium, calcium, potassium or lithium ions totally abrogated the effect of Vpr peptides, if added 30 mins before or immediately after the addition of Peptide 3. The osmotic support sorbitol also provided partial protection, and total protection was provided by incubation with 0.1 x YEPD. These results are summarised in Table 6. This apparent protection in the presence of salts is due to the inability of yeast cells to bind and internalise the peptides containing the H(S/F)RIG motif. However, in the case of mammalian cells the uptake of these peptides was observed in normal serum-containing medium.

Table 6

Negation of Effect of Peptide 3 on Yeast Colony Formation

Other Treatment	% colony formation with Peptide 3 treatment	
	plus other treatment before or immediately after peptide addition	with other treatment 30 min after peptide addition
0	0	0
0.5 M NaCl	100	3
50 mM NaCl	100	
5 mM NaCl	1	
0.8 M LiCl	80	
60 mM KCl	100	
0.1 M $CaCl_2$	100	
0.1 x YEPD	100	
0.3 M sorbitol	25	

Example 15 Viability of Bacterial Cells Producing Vpr

Cells were treated with the inducer IPTG and aliquots plated on to 2 x YT + Ampicillin plates after appropriate times, and the number of colonies was counted

after overnight incubation of the plates. The data show that the production of GST and GST-Vpr.BE (encoded by the *BamHI-EcoRI* fragment of *vpr*) does not kill *E. coli* cells, but induction slows the growth. The production of GST fused to the full-length Vpr protein leads to similar effects after three hours induction; however, after 30 hours in inducer there is an actual reduction in the number of ampicillin resistant cells/ml. This is despite the culture reaching a typical optical density after overnight incubation, and indicates that the vast majority of cells in the culture have lost their Amp^r determinant (and no longer express *vpr*). Furthermore it implicates the C-terminal region of Vpr in the cell death. It is also clear that the uninduced cells have not increased in number, indicating a cytostatic effect in the absence of inducer. However, the cells grew to 10^8 cells/ml, suggesting that the toxic effect may be specific to a particular condition, such as growth in spent medium.

Table 7

Protein produced	[Amp^r cells/ml] and time after induction				
				30 hours	
		-inducer	+inducer	-inducer	+inducer
GST	1.1×10^8	4×10^8	2.2×10^8	4.8×10^9	4.4×10^9
GST-Vpr.BE	6.8×10^7	2×10^8	4×10^7	4.8×10^9	2.2×10^9
GST-Vpr	1.3×10^8	4×10^8	1.4×10^8	1×10^8	$<2 \times 10^6$

Example 16 Interaction of Fluorescence-Labelled Peptides with Cells

Figure 19 shows the association of FITC (fluorescein isothiocyanate)-labelled peptides with $CD4^+$ human cells measured by flow cytometry following (A) electroporation, and (B) extracellular addition without electroporation.

Figure 20 shows the association of FITC-labelled peptides without electroporation in *S. cerevisiae* yeast cells measured by flow cytometry. Peptides 2-4 exhibit high degrees of association with yeast cells, while there is a considerably lesser association of Peptide 5. Peptide 1, which lacks the H(S/F)RIG motif, exhibits over one hundred-fold less association with cells than Peptide 2 and 3. By light microscopy we have observed that the FITC-labelled Peptide 3 efficiently targets into yeast and mammalian cells, as shown in Figure 21. Peptides 2 and 4 also behave similarly. These data indicate that the H(S/F)RIG motif are sufficient for intracellular targetting and they, or related derivatives that could also be a subset of the sequence, will be useful carriers for the delivery of agents into cells for treatment of diseases.

Figure 21 shows the internalised FITC-labelled peptide 3 in human $CD4^+$ cells (A) and in yeast cells (B). The FITC-labelled human cells include some cells that are still intact and others undergoing lysis.

Example 17 Genetic Interaction Between Vpr and Sac1 p and Actin

Yeast *act1* and *sac1* mutants, DBY1195 and DBY1715 respectively, were transformed with pYEULCBX and pYEULCBX.Vpr. Transformants were then induced on plates containing 0.5 mM copper sulfate to assay for the effects of the Vpr. DY150 [pYEULCBX] and DY150 [pYEULCBX.Vpr] transformants have been described previously. An example of these results for the *sac1-vpr* interaction is shown in Figure 22.

We have found that Vpr shows structural homology to the yeast protein Sac1p. The precise function of Sac1p in assembly of the actin cytoskeleton of yeast cells is still under investigation; however, *sac1* mutants display profound cytoskeletal defects and growth arrest at low temperature (Cleves *et al*, 1989; Novick *et al*, 1989; Whitters *et al*, 1993). Production of Vpr in yeast possibly

causes similar effects to *sac1* mutants, due to sequence and functional similarity between Sac1p and Vpr; the production of Vpr could compete with normal Sac1p function and lead to cytoskeletal defects, including gross cell size and ultimate growth arrest. Indeed, in the many studies of yeast with cytoskeletal defects, mother cells are abnormally large and daughter cells are abnormally small (see for example Liu and Bretscher, 1992). We have found that time-lapse analysis of newly-induced cells producing Vpr shows the same phenomenon. Osmosensitivity also indicated possible cytoskeletal defects induced by Vpr. Large cells producing Vpr were isolated by flow cytometry and plated onto media containing high osmotic strength and normal media. Only 50% of the cells capable of growth on normal medium could grow on high osmotic strength medium, indicating structural defects in those cells.

Vpr appears to produce cytoskeletal defects in mammalian cells as well as in yeast cells. Work by Levy and colleagues showed that in a rhabdomyosarcoma cell line Vpr produced cell replication arrest and gross cell enlargement (Levy *et al*, 1993). Furthermore, in a $CD4^+$ T-lymphoblastoid cell line it was shown that HIV-1 caused ultrastructural changes, including membrane disruption, "ballooning" and vacuolisation of the endoplasmic reticulum, during the first hour of infection (Fermin and Garry, 1992). These data are consistent with cytoskeletal defects, and investigation of the cytoskeleton in those cells would be of interest.

What is the role of Vpr in the HIV-1 life cycle, and is induced growth arrest relevant to this? For some time there has been a dilemma regarding the distinction between HIV-1 and other retroviruses: retroviruses usually require cell proliferation for infection, while HIV-1 infects non-proliferating cells such as terminally-differentiated macrophages. Lewis *et al* (1993) showed that $CD4^+$ cell lines can be productively infected with HIV-1 when they are arrested in G2 growth phase. Non-

proliferation of host cells could therefore be an initial requirement for a productive infection of all or some cell types. The function of Vpr may be to bring about growth arrest so that a process like integration may occur. If this were so, it would account for Vpr (and Vpx counterparts) being virion-associated, so that early events can be initiated. Antibodies to Vpr have been detected in only 17% of AIDS patients, but are found in 47% of asymptomatic individuals (Wong-Staal *et al*, 1987), suggesting that the Vpr is present early in infection, and therefore that it is probably essential only at that time. It also follows then that inhibitors of Vpr should prevent infection or slow extracellular spread of the virus.

In this study we have shown that portions of Vpr containing the sequence HFRIGCRHSRIG or even RHSRIG cause cell damage and irreversible growth arrest when added to yeast cells. This effect is related to cell number and peptide concentration, and it appears that a minimum of 10^6 molecules of peptide per cell is required to observe the effect. We have also shown that the same sequence was involved in causing osmosensitivity and structural defects when peptides containing this sequence were electroporated into cells. Only intracellular effects were examined after electroporation, since YEPD in the electroporation medium abrogated the extracellular effect.

Peptides containing H(S/F)RIG motifs appear to cause cell damage resulting in increased permeability and cell lysis in both mammalian and yeast cells. We have observed that Vpr peptides containing the H(S/F)RIG motif are cell associated; fluorescence microscopy using FITC-labelled peptides indicates that they are internalised within 1 h. We have ascertained that the H(S/F)RIG motif causes active uptake of the peptide; uptake of FITC-labelled Peptide 1 appeared to be one hundred-fold lower than that of peptides containing the H(S/F)RIG motif, suggesting that the motif does promote uptake. In Example 7, peptide uptake was artificially obtained by

electroporation, and the outcome studied was osmosensitivity; the electroporation technique itself led to variable degrees of cell death or loss of colony-forming ability. However, among the numerous cells that formed colonies there was a high degree of osmotic sensitivity. The effects observed in Examples 10 to 15 are quite different, with total loss of colony forming ability. These differences may be related to the localisation of the peptide within the cell. Osmosensitivity, rather than loss of colony forming ability, was also observed with the expression of the *vpr* gene in yeast in a more life-like situation, as described in Example 2.

What AIDS phenomenon then can be correlated with our results? Recent studies by Levy *et al* (1994) show that Vpr does exist in the serum, suggesting that it is released from infected cells, and indicating that in designing putative antagonists of the protein it is relevant to consider the extracellular effects of Vpr, which may be responsible for the killing by HIV of uninfected host cells, as well as its intracellular effects.

Using biologically active fragments of Vpr, we have shown that parts of Vpr, and presumably the entire Vpr protein, irreversibly affect colony-forming ability via the action of the H(S/F)RIG motifs within Vpr. The mode of action of this effect may be related to the Ca^{2+} ion channel, since the Ca^{2+} ion channel blocker TMB-8 abrogates the effect, as shown in Examples 14 and 15.

References cited herein are listed on the following pages, and are incorporated herein by this reference.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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CLAIMS

1. An antagonist of the Vpr protein of human immunodeficiency virus (HIV), or of a biologically active fragment or analogue thereof, comprising at least one amino acid sequence motif selected from HFRIG and HSRIG, said antagonist having the ability to inhibit one or more activities mediated by Vpr, selected from the group consisting of growth arrest, cell replication arrest, cytotoxicity, cytoskeletal disruption, and effects on the endoplasmic reticulum.

2. An antagonist according to Claim 1, wherein the Vpr protein further comprises at least one sequence selected from the group consisting of HSRIS, HFRAG, HIRAG, HLRAG, RSRKG, RSRIS and RSRIG.

3. An antagonist according to Claim 1 or Claim 2, wherein the Vpr protein is a fragment comprising at least the C-terminal 21 amino acids of the Vpr sequence.

4. An antagonist according to Claim 3, wherein the Vpr protein is a fragment comprising at least the C-terminal 33 amino acids of the Vpr sequence.

5. An antagonist according to any one of Claims 1 to 4, selected from the group consisting of an antibody, an anti-sense RNA, and a triple-stranded DNA.

6. An antagonist according to Claim 5, which is an antibody.

7. An antagonist according to Claim 6, which is a monoclonal antibody.

8. An antagonist according to Claim 5, which is an anti-sense RNA.

9. An antagonist according to Claim 5, which is a triple-stranded DNA.

10. A pharmaceutical composition comprising as active component an antagonist according to any one of Claims 1 to 9, together with a pharmaceutically-acceptable carrier.

11. A method of screening compounds suspected of being useful as antagonists of Vpr protein, or of a biologically active fragment or analogue thereof,

comprising the step of measuring the effectiveness of a test compound in inhibiting the activity of Vpr in an assay of a biological activity selected from the group consisting of growth arrest, cell replication arrest, cytotoxicity, cytoskeletal disruption, and effects on the endoplasmic reticulum.

12. A vaccine for prevention of HIV infection or for alleviation of the effects of HIV infection, comprising human immunodeficiency virus-1 or human immunodeficiency virus-2 from which the portion of the HIV genome encoding at least the C-terminal 21 amino acids of the Vpr sequence has been deleted, together with a pharmaceutically-acceptable carrier.

13. A vaccine according to Claim 12, wherein the portion of said genome encoding at least the C-terminal 33 amino acids of the Vpr sequence has been deleted.

14. A vaccine according to Claim 12 or Claim 13, wherein both a portion of the genome encoding the C-terminal region of the Vpr sequence and the portion of the HIV genome encoding the N-terminal of the Nef gene have been deleted.

15. A vpr gene in which the region encoding C-terminal portions of the Vpr protein has been replaced by an inhibitory antisense sequence or by a sequence which encodes an inhibitory peptide.

16. A method of treatment of HIV infection, comprising the step of administering to a subject in need of such treatment an effective amount of an antagonist of Vpr protein, or of a biologically active fragment or analogue thereof, as defined in any one of Claims 1 to 9, thereby to prevent HIV infection, to prevent progression of HIV infection to symptomatic AIDS, or to alleviate the symptoms of AIDS.

17. A method of treatment of a disease mediated by cell proliferation, comprising the step of administration to a mammal in need of such treatment of an effective amount of Vpr protein, or of a biologically active fragment

or analogue thereof comprising at least one amino acid sequence motif selected from HFRIG and HSRIG, thereby to inhibit proliferation of cells mediating the said disease.

18. A method according to Claim 17, wherein the disease mediated by cell proliferation is a cancer, a leukaemia, or psoriasis.

19. A method according to Claim 17 or Claim 18, wherein the Vpr protein or fragment or analogue thereof is used in conjunction with an enhancer of Ca^{2+}-channel transport.

20. A method of treatment of a disease caused by a pathogen, comprising the step of administering to a mammal in need of such treatment of an effective amount of Vpr protein, or of a biologically active fragment or analogue thereof comprising at least one amino acid sequence motif selected from HFRIG and HSRIG.

21. A method according to Claim 20, wherein the disease is caused by a bacterium, a parasite, a yeast or a fungus.

22. An agent for delivery of a pharmaceutically active substance to a cell membrane or to the interior of a cell, comprising a Vpr peptide or a biologically active fragment or analogue thereof comprising at least one amino acid sequence motif selected from HFRIG and HSRIG, said peptide, fragment or analogue being linked to said pharmaceutically active substance.

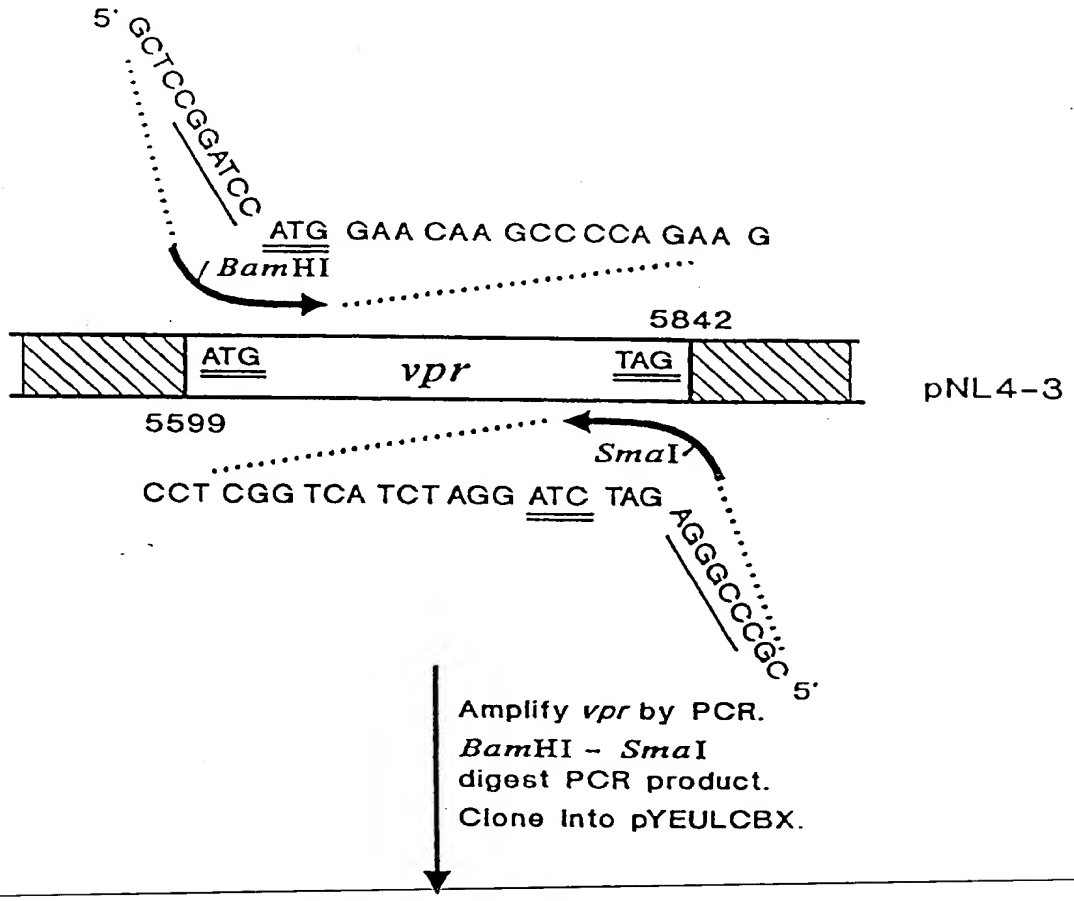
23. A method of delivery of a pharmaceutically active substance to a cell membrane or to the interior of a cell, comprising the step of contacting said cell with an agent as defined in Claim 23.

24. Use of Vpr protein, or of a biologically active fragment or analogue thereof comprising at least one amino acid sequence motif selected from HFRIG and HSRIG in the treatment of a disease mediated by cellular proliferation.

25. Use of Vpr protein, or of a biologically active fragment or analogue thereof comprising at least one amino acid sequence motif selected from HFRIG and HSRIG in the

treatment of a disease caused by a pathogen.

26. An antagonist of the Vpx protein of human immunodeficiency virus-2 (HIV-2) or of a biologically active fragment or analogue thereof, comprising at least one amino acid sequence motif selected from the group consisting of HCKKG, CLGEG, CLGEE, CLGGE, CLGRG, HVRKG, HYTKG, HFKRG, HFKKG, HAKRD, CLQEG, CLGGG, CRGEG, CWGED, HFRCG and RRQPF, said antagonist having the ability to inhibit one or more activities mediated by Vpx, selected from the group consisting of group arrest, cell replication arrest, cytotoxicity, cytotoskeletal disruption and effects on the endoplasmic reticulum.



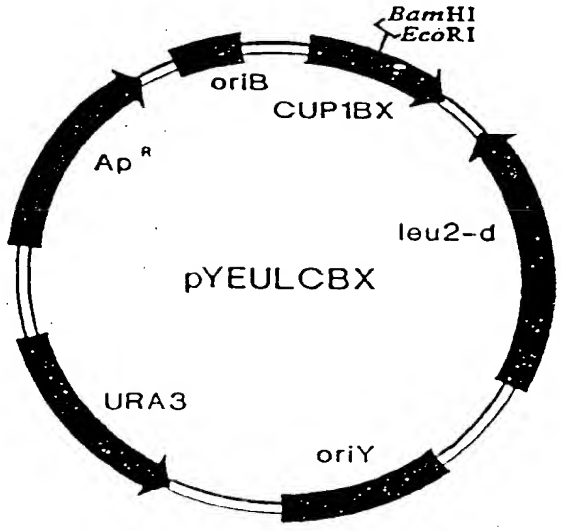


FIGURE 1

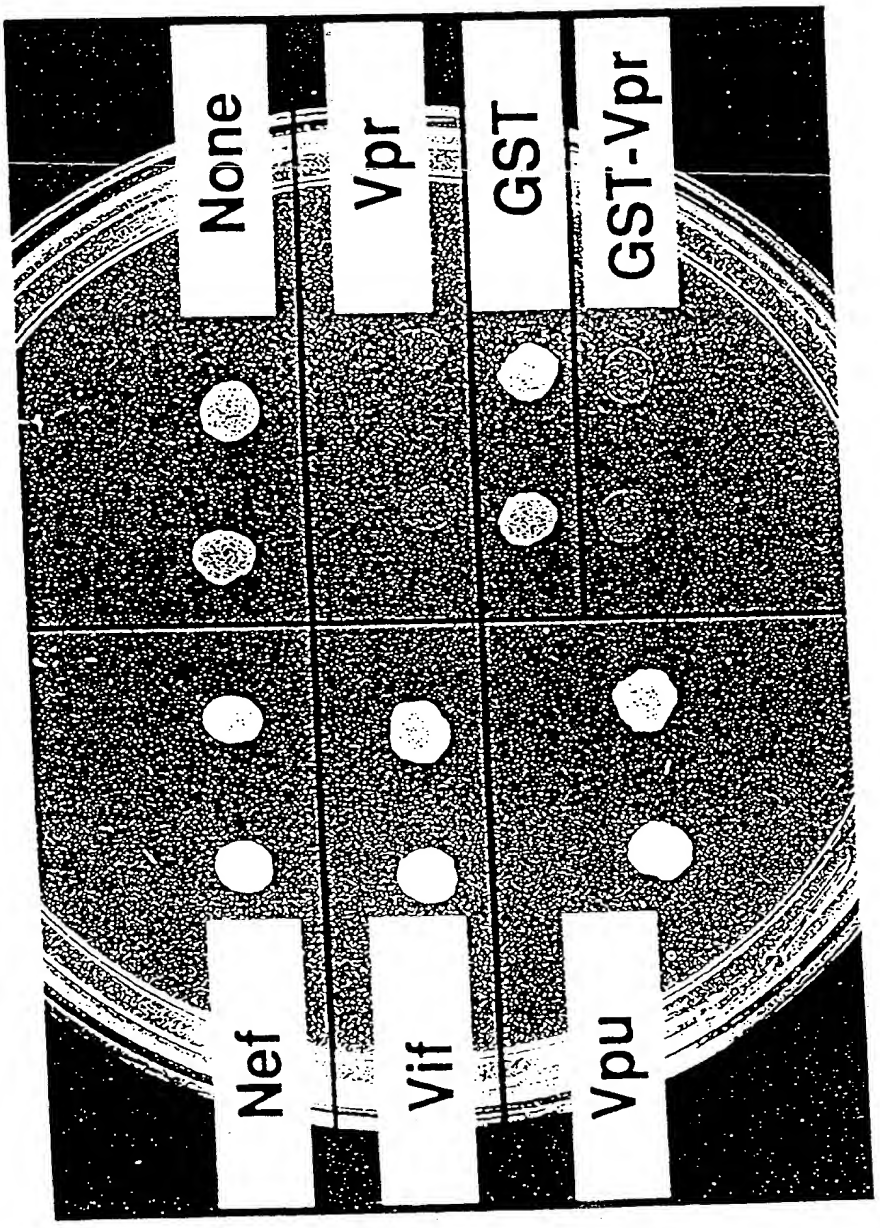


FIGURE 2

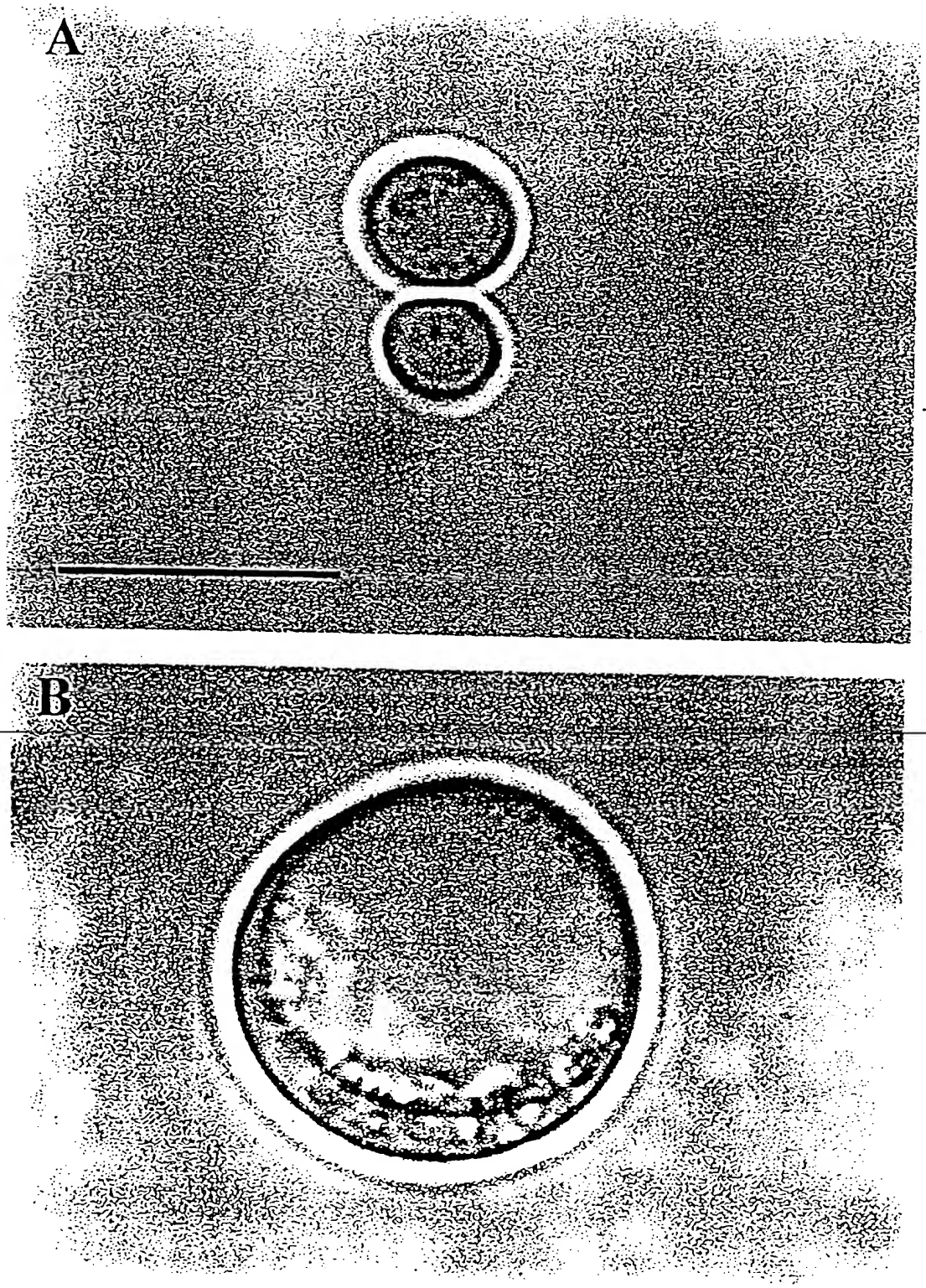


FIGURE 3

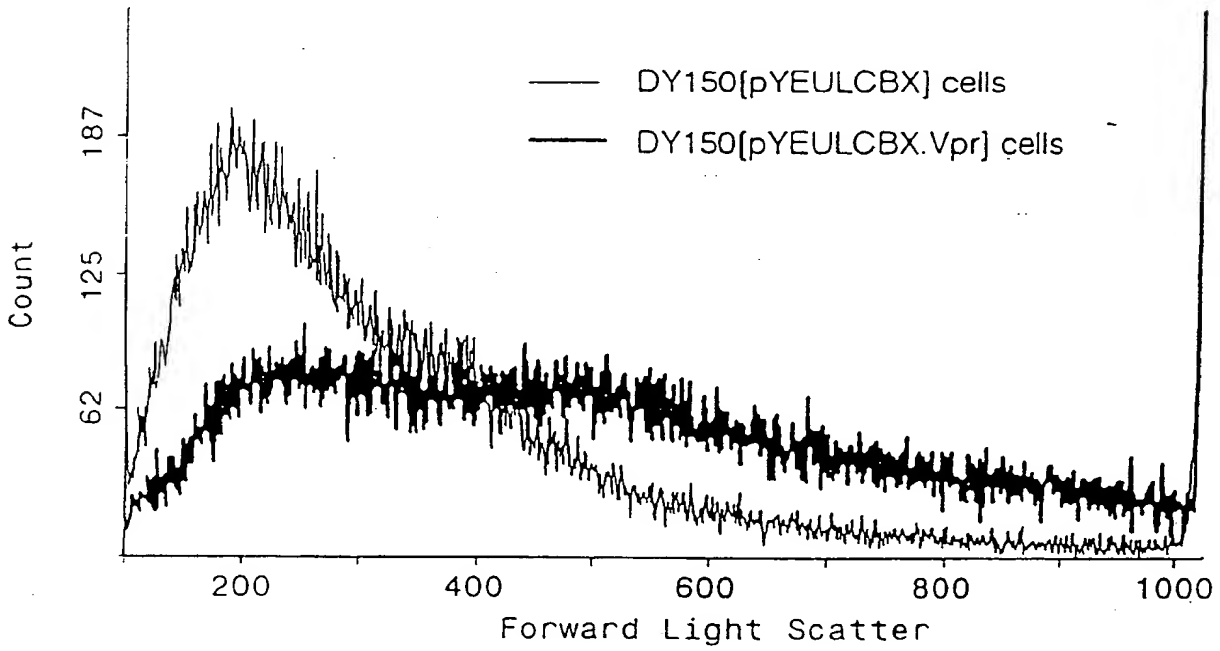


FIGURE 4

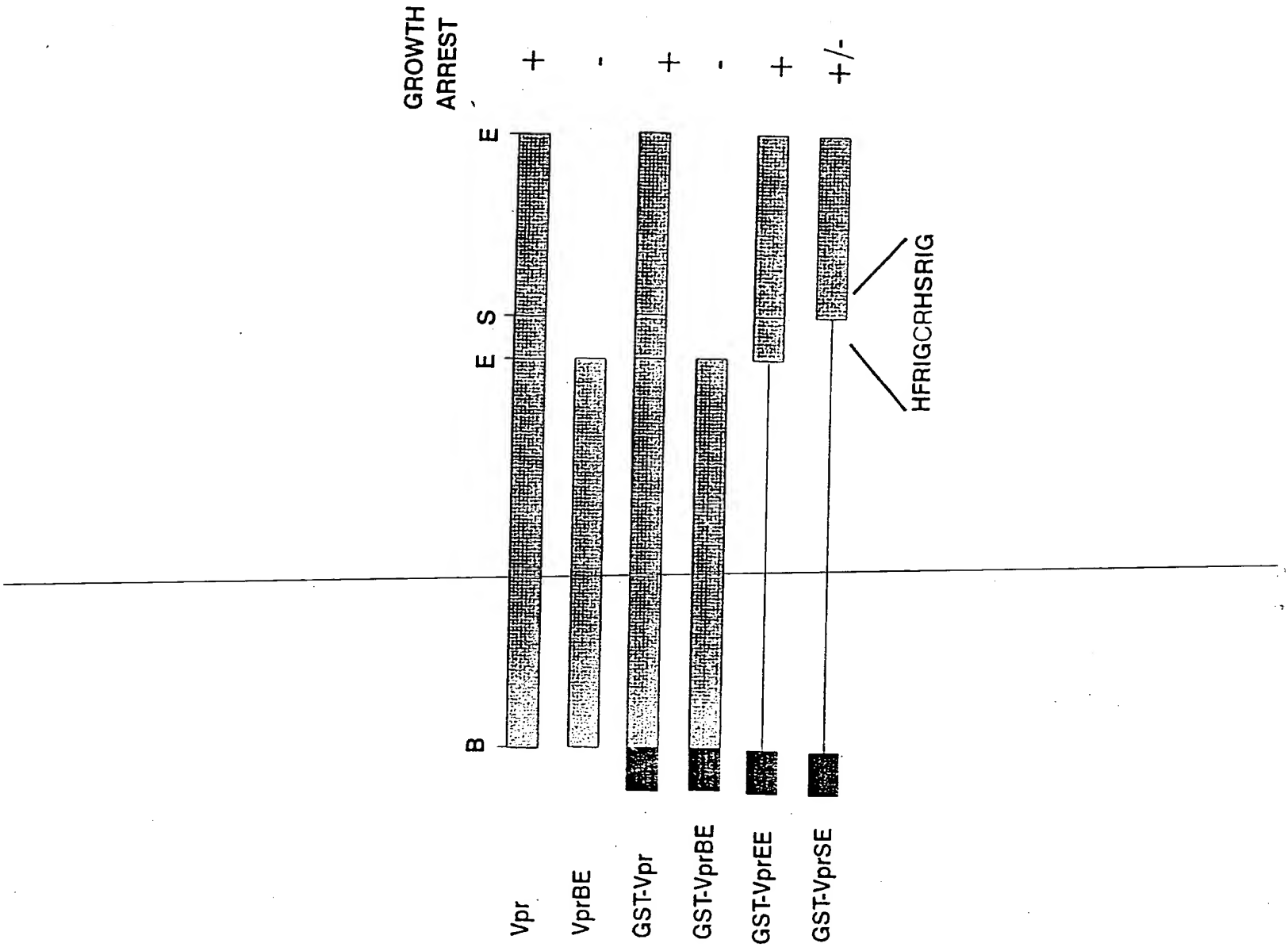


FIGURE 5

Alignment of Vpr-relatives

A.

```
HIV-1 Vpr        MEQ-APEDQG-PQREPYNEWTLELLEELKSEAVRHF-PRIWLHNLGQ--HIY  47
SIVmac Vpr     MEER-PPENEG-PQREPWDEWVVEVLEELKEEALKHFDPR-LLTALGN--HIY  48
HIV-2 Vpr  MAEAPTE-LPPVDGTL--REPGDEWIIEILREIKEEALKHFDPR-LLIALGK--YIY  51
HIV-2 Vpx   MTDPRETVPPGNSGEETIGEAFAWLNRTVEAINREAVNHL-PRELIFQVWQRSWRY  55
SIVmac Vpx  MSDPRERIPPGNSGEETIGEAFEWLNRTVEEINREAVNHL-PRELIFQVWQRSWEY  55

HIV-1 Vpr    --ETYG-DTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGA--SRS    96
SIVmac Vpr   --NRHG-DTLEGAGELIRILQRALFMHFRGGCIHSRIGQPGGGNPLSAIPPSRSML 101
HIV-2 Vpr    --TRHG-DTLEGARELIKVLQRALFTHFRAGCGHSRIGQTRGGN-LSAIPTPRNMQ 103
HIV-2 Vpx    WHDEQGMSESYTKYRYLCIIQKAVYMHVRKGCTCLGRGHGPGGWRPGPPPPPPGLV 111
SIVmac Vpx   WHDEQGMSQSYVKYRYLCLMQKALFMHCKKGCRCLGEGHGAGGWRPGPPPPPPGLA 111
```

B.

```
Sac1p        ...SLQRNEKVGPAASWKTADERFFWNHYLTEDLRNFA-HQDPRI-DSFIGQPVIY 126
HIV-1 Vpr       MEQAPEDQGP--QREPYNE---WTLELLEELKSEAVRHFPRIWLHNLGQ-HIY  47

Sac1p         -GYAKTVDAVLNATPIVLGLITRRSIFRAGTRYFRRGVDKDGNVGNFNETE...  176
HIV-1 Vpr    ETYGDTWAGVRAIIRILQQLL--FIHFRIGCRHSRIGVTRQRRARNGASRS      96
```

FIGURE 6

SUBSTITUTE SHEET (Rule 26)

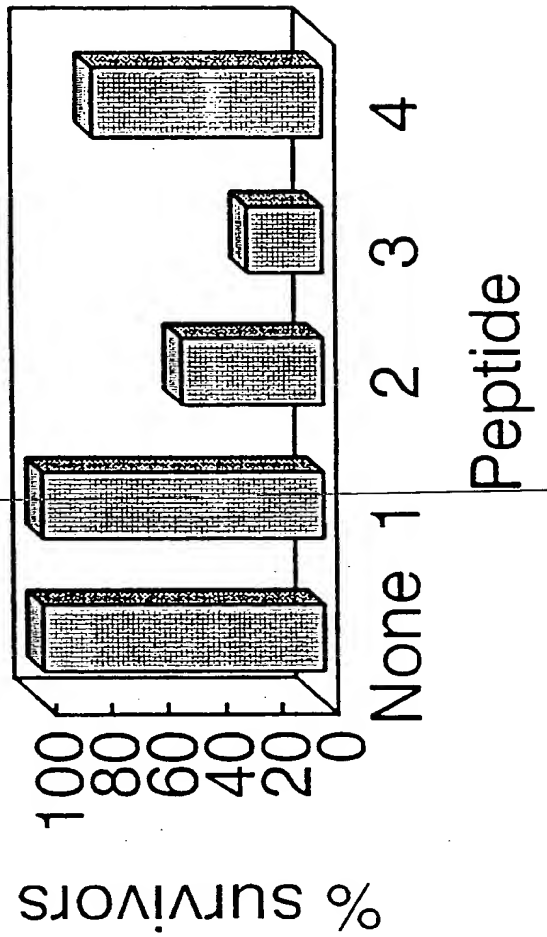


FIGURE 7

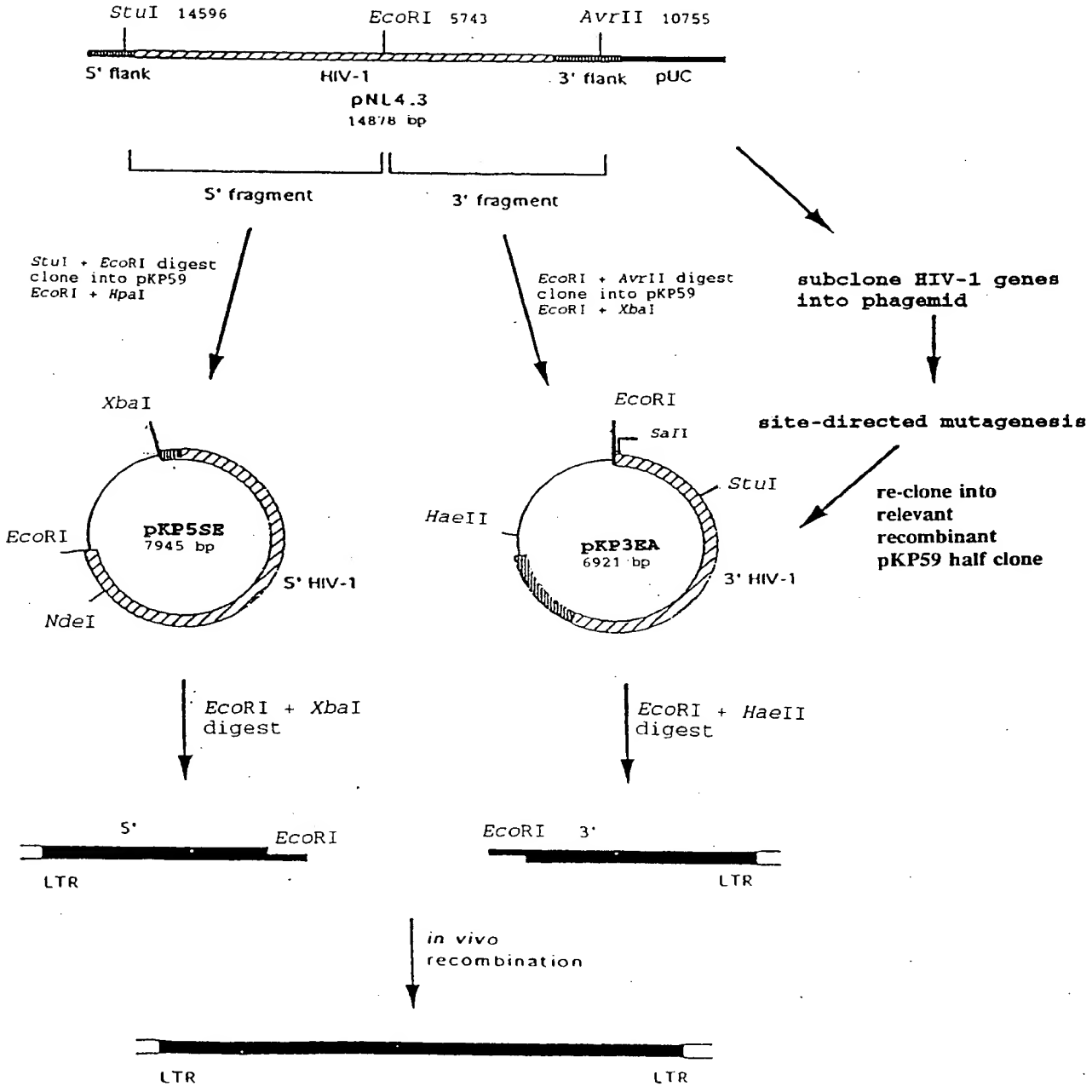


FIGURE 8

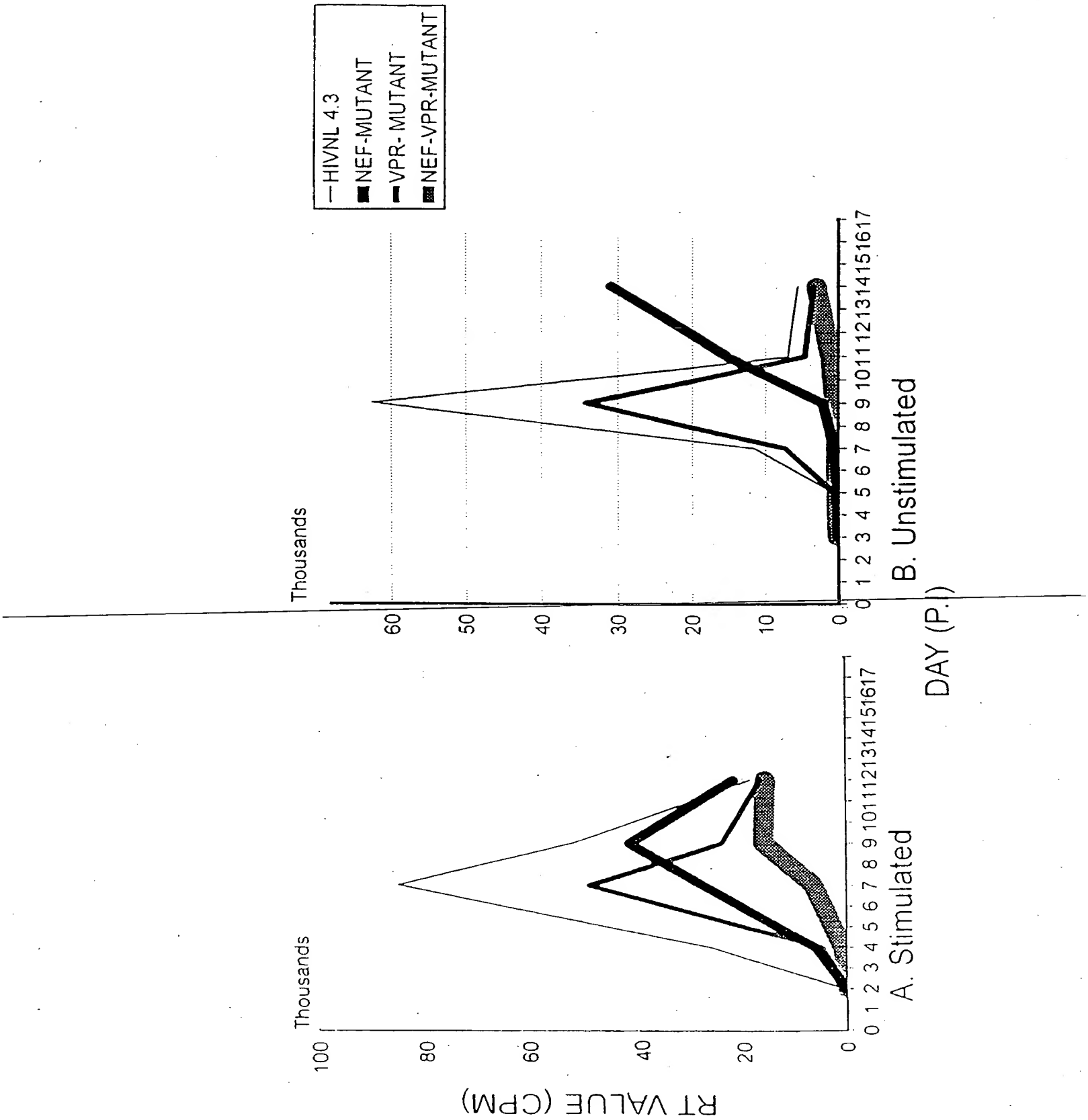


FIGURE 9

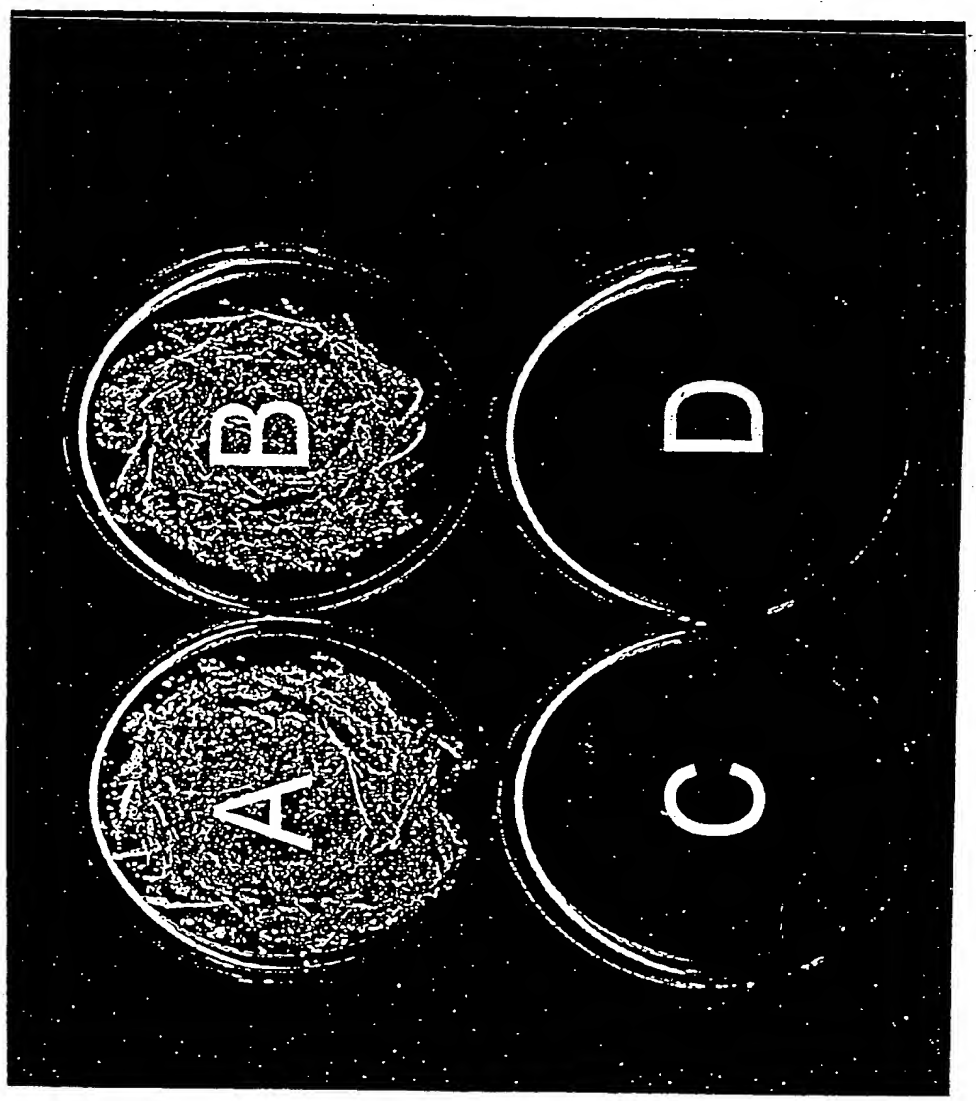


FIGURE 10

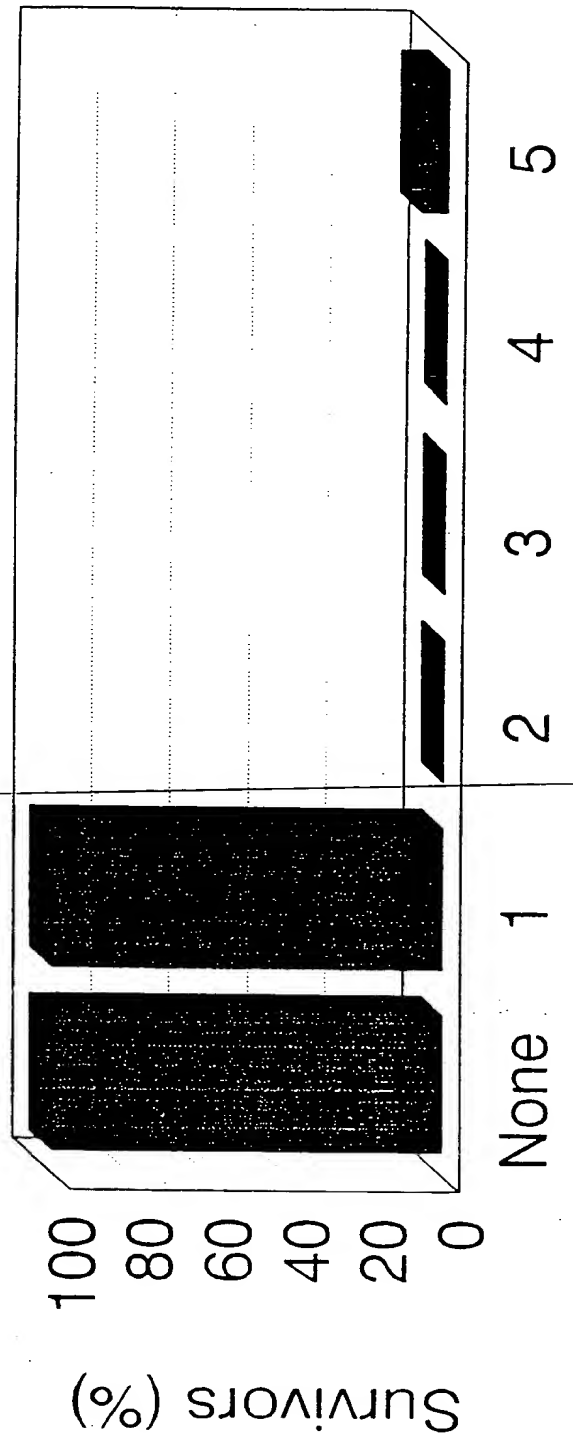


FIGURE 11

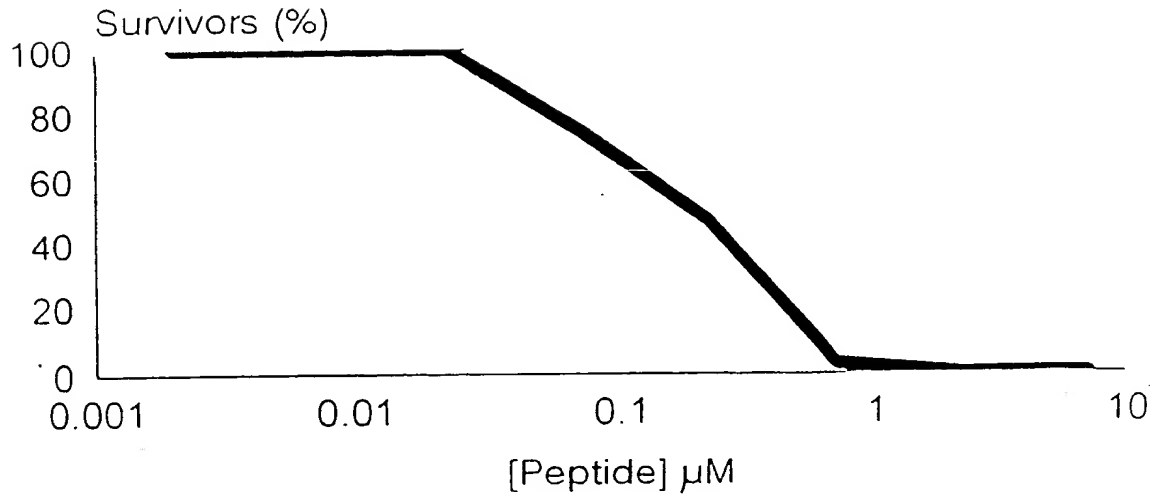
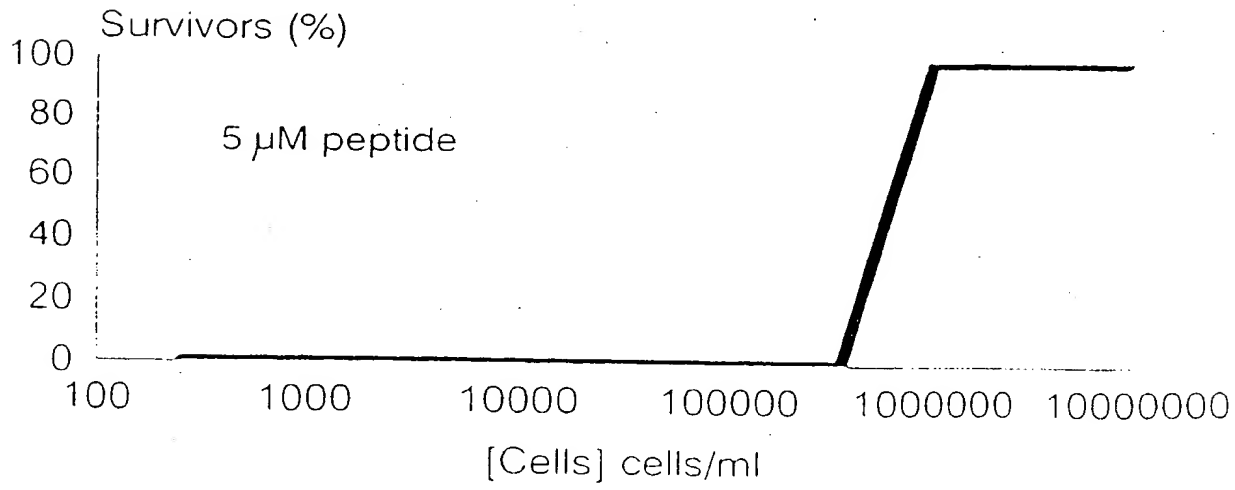


FIGURE 12



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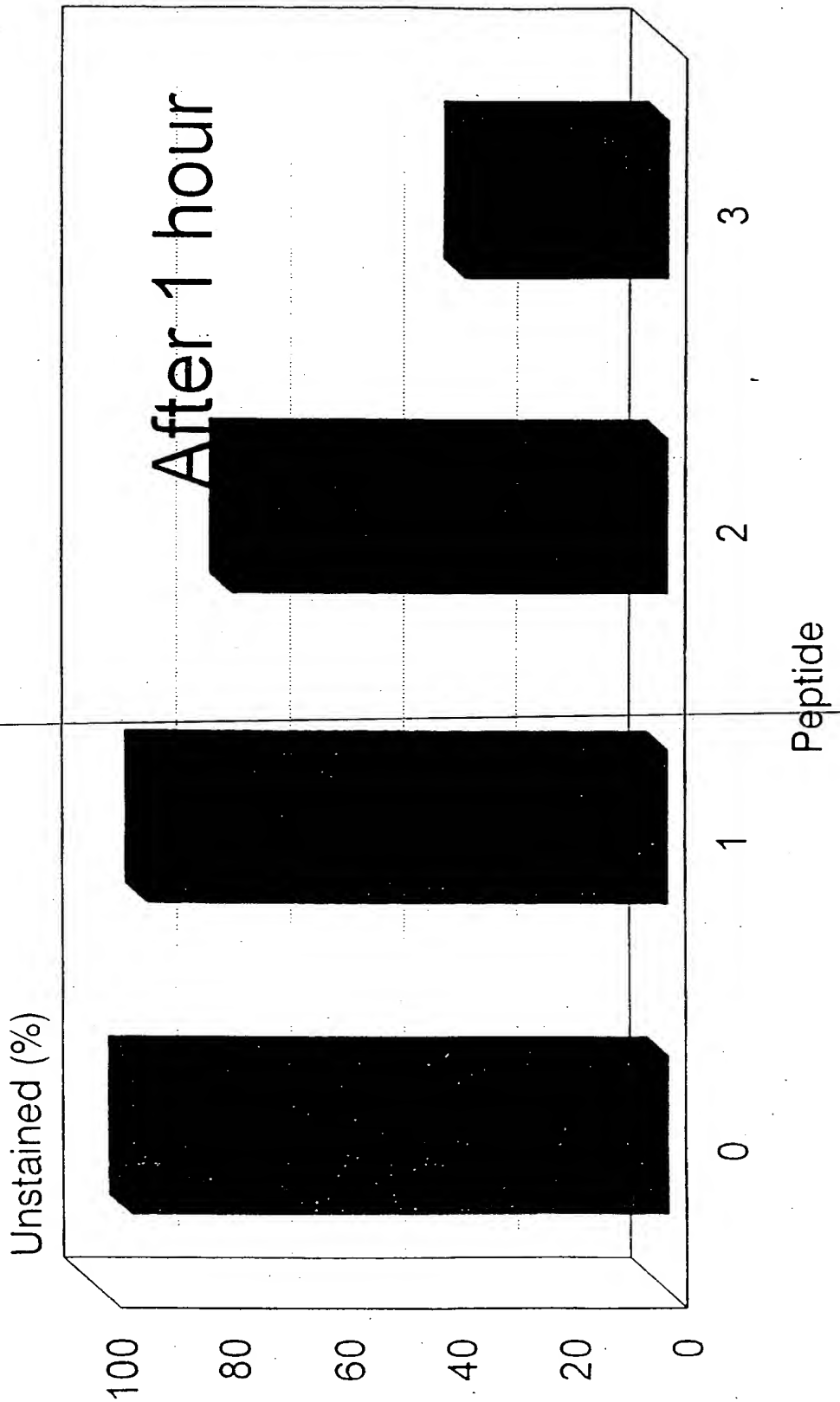


FIGURE 14

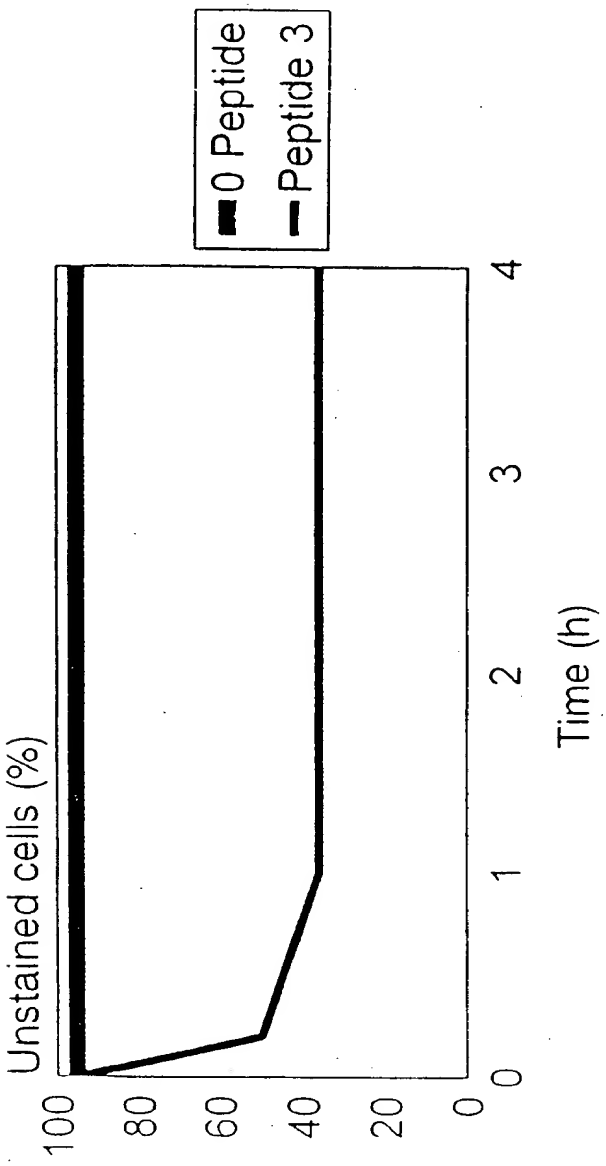


FIGURE 15

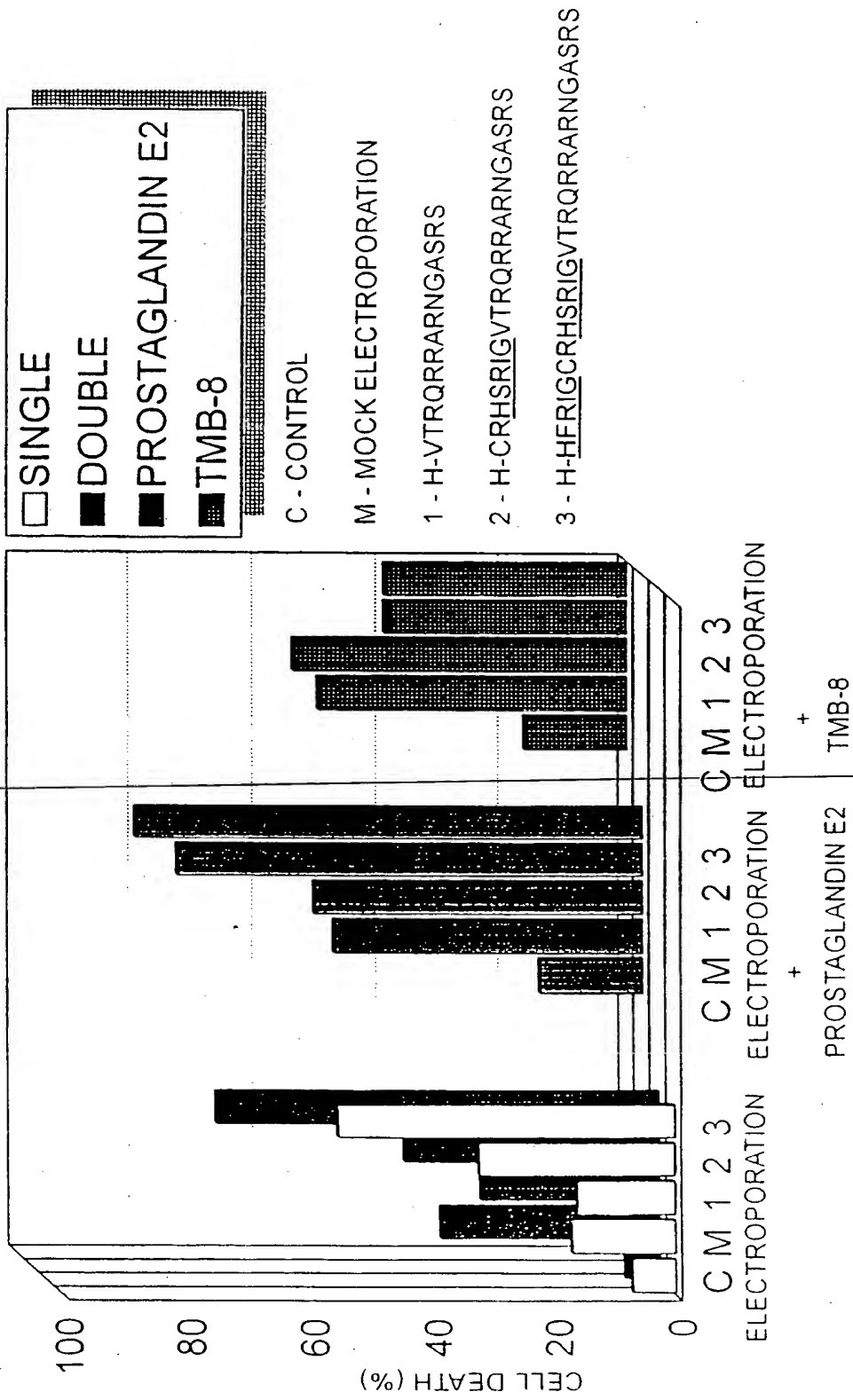
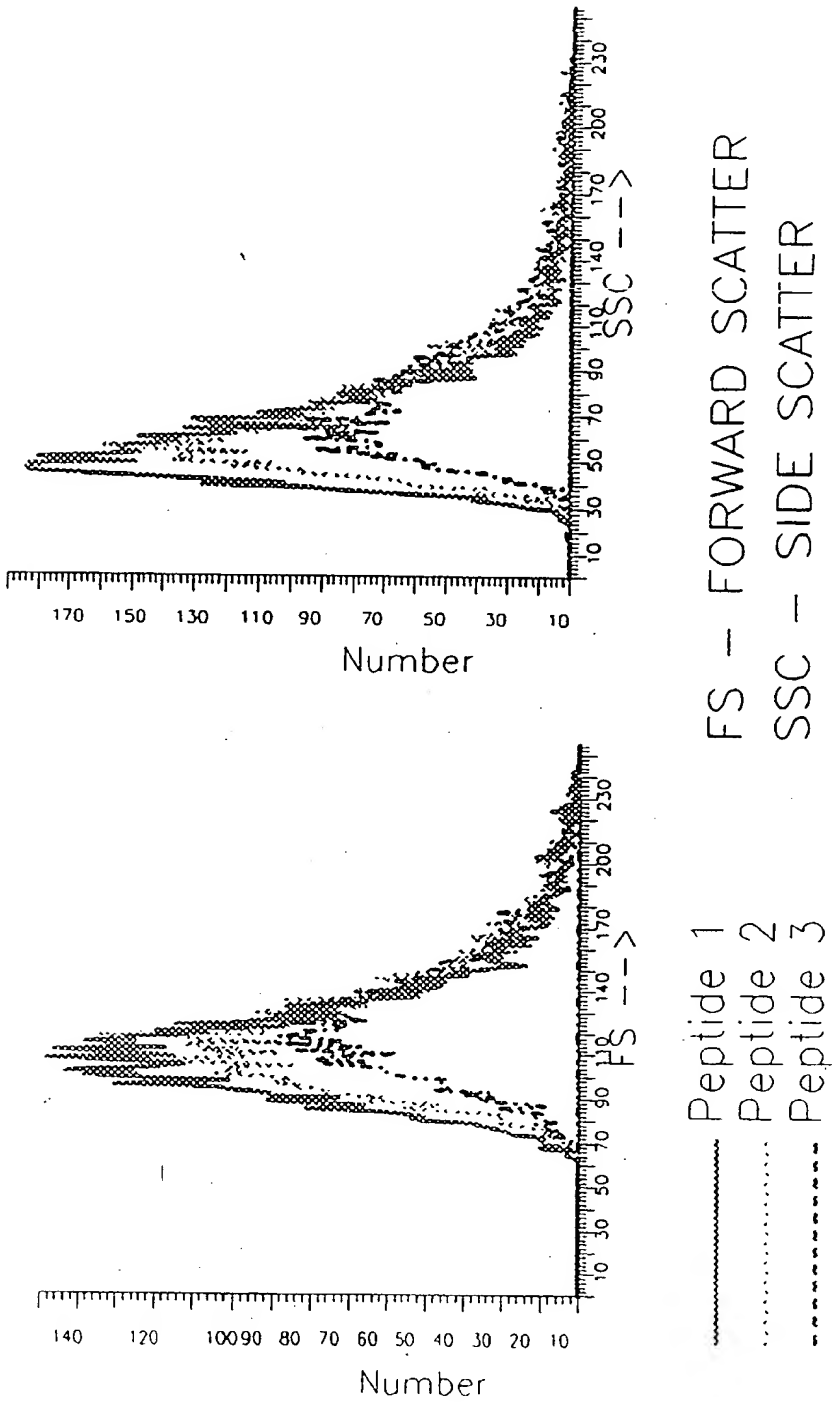


FIGURE 16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170 150 130 110 90 70 50 30 10
Number
FS -->
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140 120 100 90 80 70 60 50 40 30 20 10
Number
FS - FORWARD SCATTER
SSC - SIDE SCATTER
Peptide 1
Peptide 2
Peptide 3

FIGURE 17

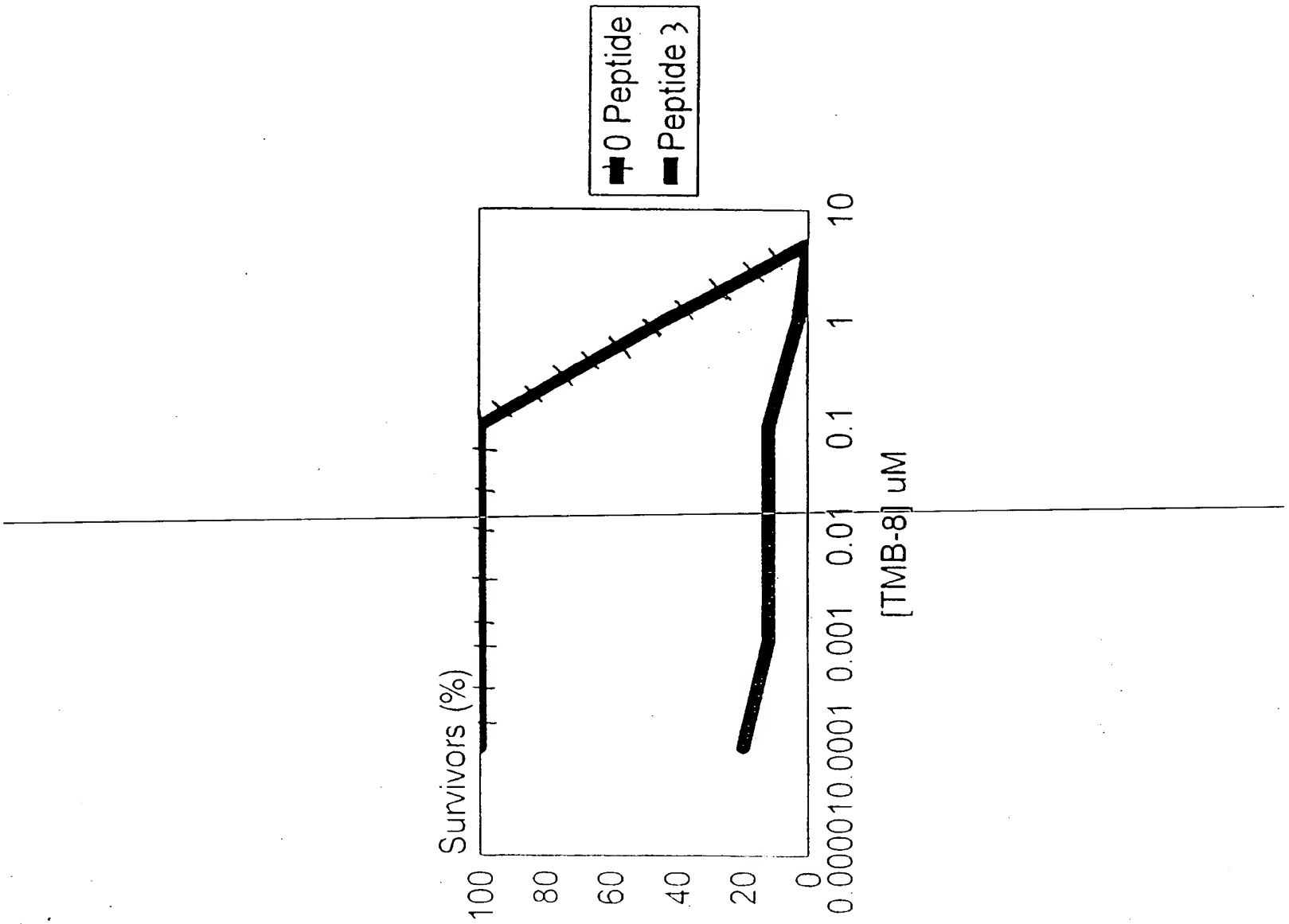
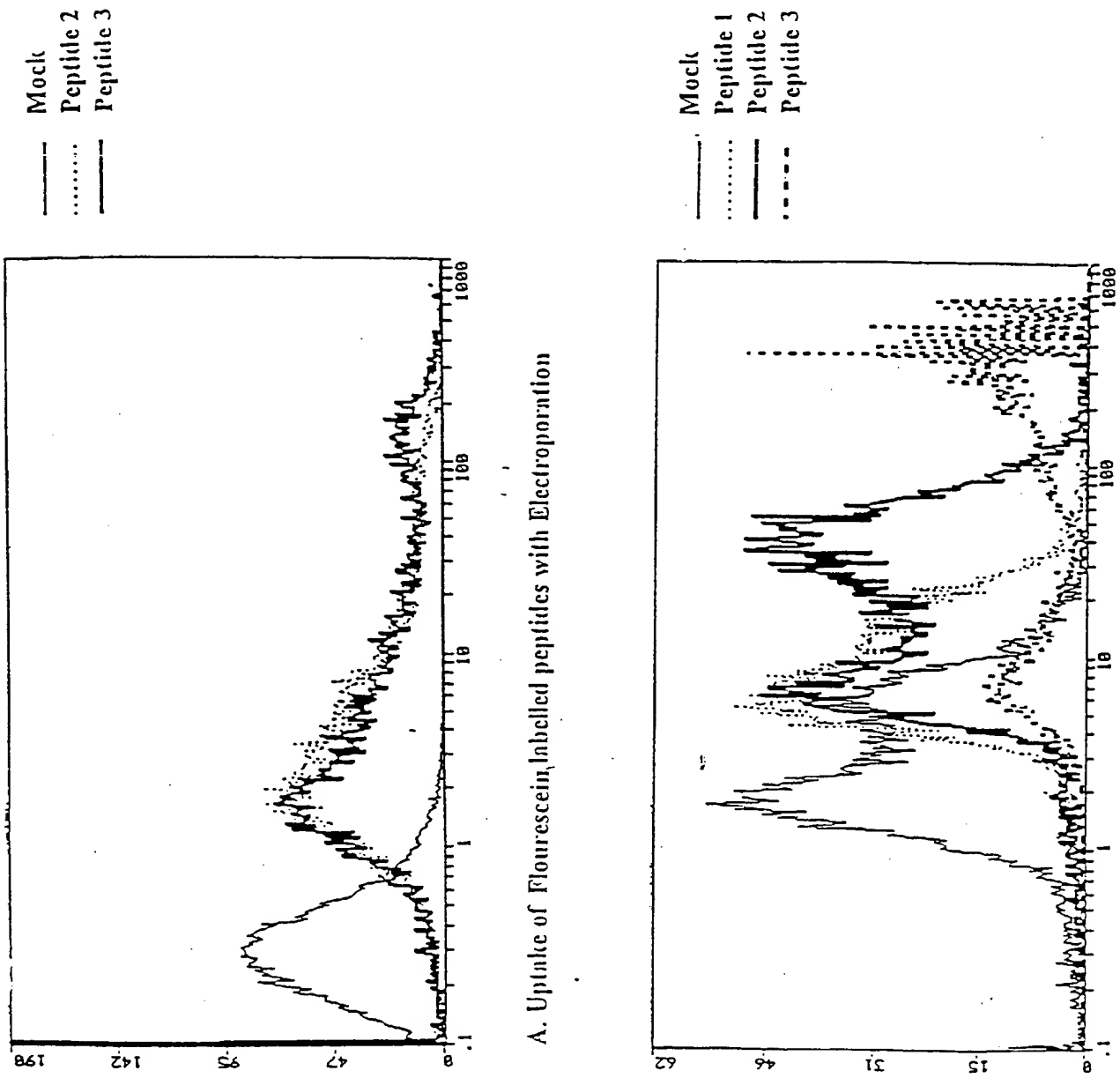


FIGURE 18



A. Uptake of Flourescein labelled peptides with Electroporation

FIGURE 19

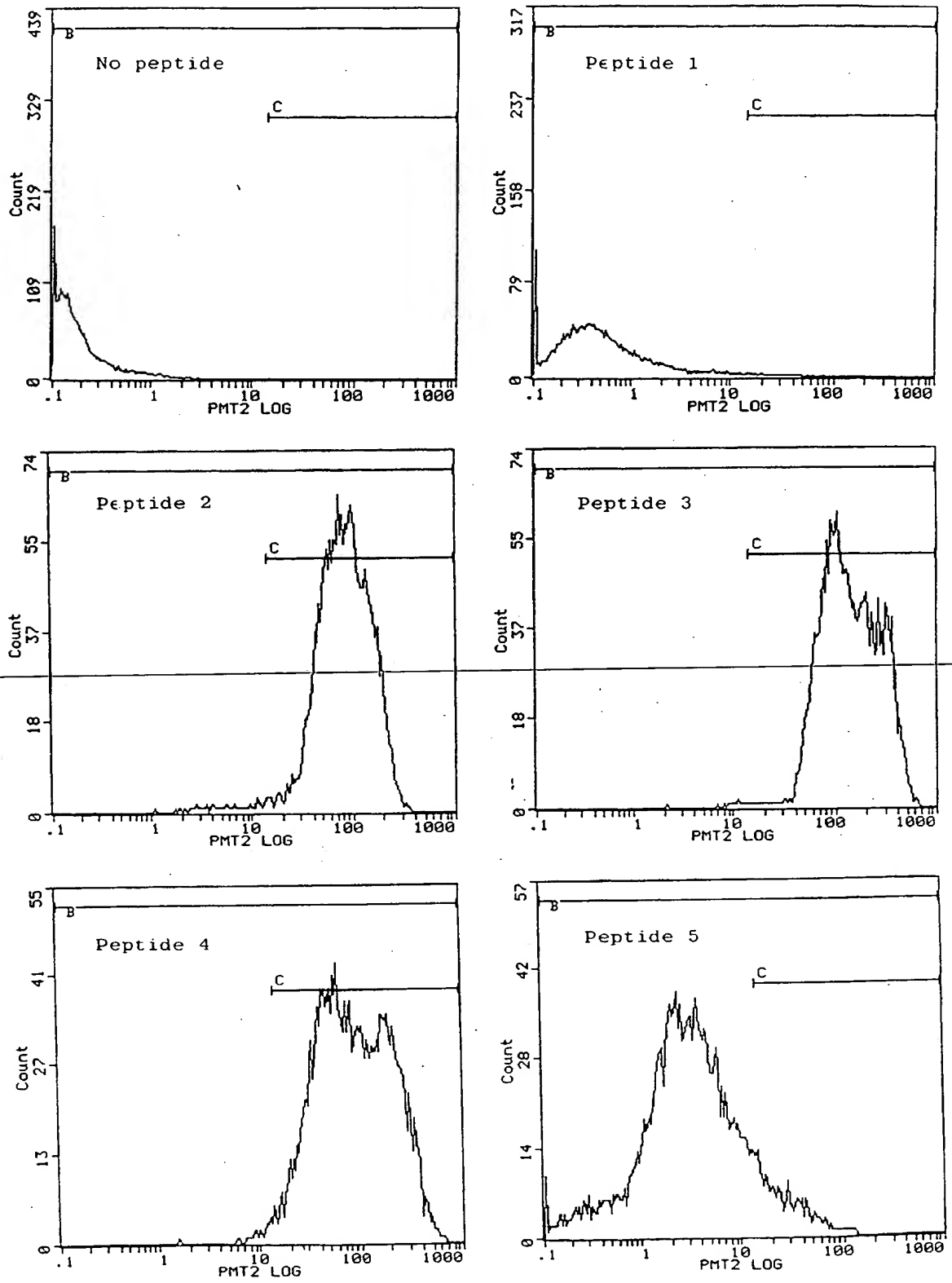
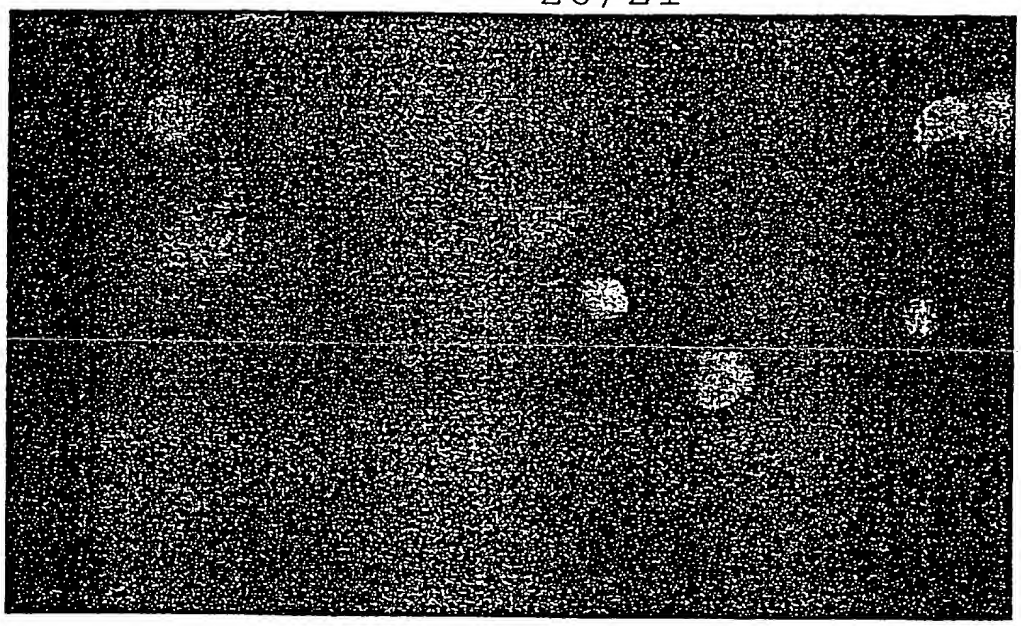


FIGURE 20



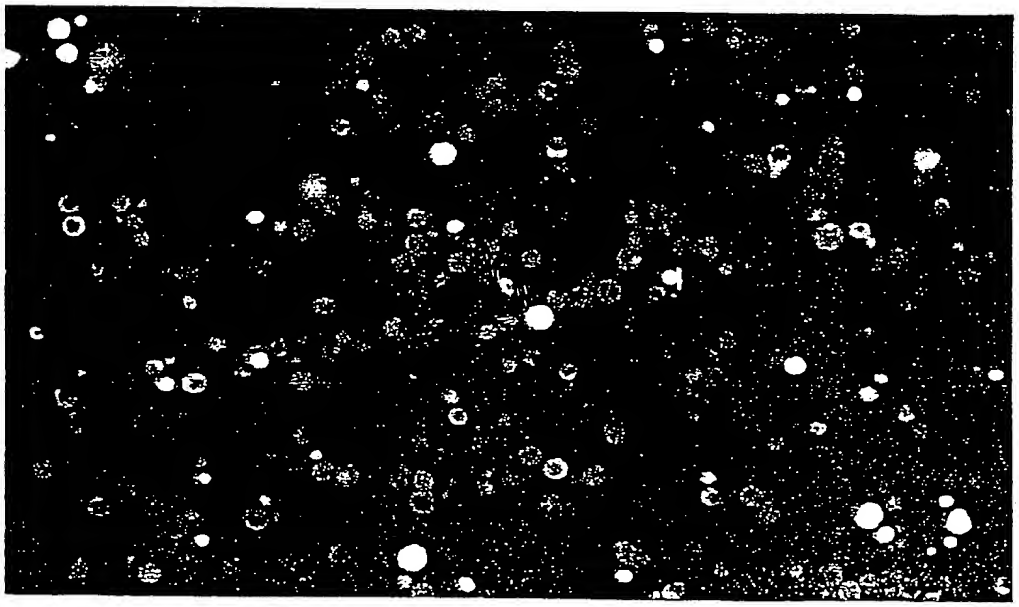


FIGURE 21

	pYEULCBX		pYEULCBX.Vpr	
Strain	0 Cu	0.5 mM Cu	0 Cu	0.5 mM Cu
Wild-type (DY150)	++++	++++	++	-
act1 (DBY1195)	++++	++++	++++	+++
sac1 (DBY 1715)	++	++	++	++

Vpr interacts with Sac1p

Strain	Plasmid	0 Cu	+ Cu
w.t.	pYEX-BX		
	pYEX-BX-Vpr		
sac1	pYEX-BX		
	pYEX-BX-Vpr		

FIGURE 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 95/00169

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[6] C07K 7/06, 7/08, 14/155, 14/16, 16/10; C07H 21/04; C12N 15/63, 15/48; C12Q 1/02; A61K 39/21, 39/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : FILE WPAT: Keywords: See below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : IPC : C07K 7/06, 7/08, 7/10, 15/12, 15/28, C07H 21/04, C12N 15/63, 15/48, C12Q, 1/02

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
FILE WPAT: Keywords: VIRAL PROTEIN R, VIRAL PROTEIN X, VPR, VPX
FILE CASM: Keywords: as above and HIV or HUMAN IMMUNODEFICIENCY VIRUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	WO,A, 9200987 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 23 January 1992 See entire document; claim 9, 36, 46; page 4 line 26, pages 16 to 20	12-14
X	Journal of Experimental Medicine, Vol 172, september 1990, Choppin et al "Analysis of Physical Interactions between Peptides and HLA molecules and Application to the Detection of HIV-1 Antigenic Peptides" pages 889-899 See entire document; p 896 column 2 line 7; table 1 Vpr 68-80 (HFRIG)	1-7

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 June 1995	3 JULY 1995 (03.07.95)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer MARK ROSS Telephone No. (06) 2832295

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	International Journal of Peptide and Protein Research, Volume 36, No 3, (1990) Gras-Masse et al "A synthetic protein corresponding to the entire vpr gene product from HIV-1 is recognised by antibodies from HIV-infected patients" pages 219-226 See entire document; Fig 1, page 221 column 1 paragraph 5 page 224	1-7
X	Journal of Virology, Volume 67 No 2 (1993) Lang et al "Importance of vpr infection of Rhesus Monkeys with Simian Immunodeficiency Virus" pages 902-912 See entire document; page 908, column 2 last paragraph	1-7
X	Journal of Virology, Vol 67 No 7 (1993) Balotta et al "Antisense Phosphorothioate Oligo deoxynucleotides targeted to the vpr gene inhibit HIV-1 replication in primary human macrophages" pages 4409-4414 See entire document	1-5, 8, 11, 16
X	WO,A, 9403596 (UNIVERSITY OF HAWAII) 17 February 1994 See entire document; claims 12 and 43	1-5, 8, 16
P,X	AU,A, 62524/94, (WEINER et al) 14 September 1994 See entire document; claims 2, 6, 14, 32	1-7, 10-14, 16-25
P,X	AU,A, 58487/94 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 29 August 1994 See entire document	12-14
P,X	Journal of Acquired Immune Deficiency Syndromes, Vol 7 (July 1994) Sarobe et al "Induction of neutralising antibodies against HIV-1 using synthetic peptide constructs containing an immunodominant T-Helper Cell determinant from vpr See entire document; abstract (HFRIG)	1-7, 12-14

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	9015875	EP	474797	JP	4506605
WO	9200987	WO	9217945	US	5175872
AU	62524/94	IL	108707	WO	9419456
AU	58487/94	WO	9417825		

END OF ANNEX